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(54) Title: THERAPY OF CENTRAL NERVOUS SYSTEM BY GENETICALLY MODIFIED CELLS

(57) Abstract

Methods of genetically modifying donor cells by gene transfer for grafting into the central nervous system to treat defective, diseased or damaged cells are disclosed. The modified donor cells produce functional molecules that effect the recovery or improved function of cells in the CNS. Methods and vectors for carrying out gene transfer and grafting are described.

DONOR CELL

VECTOR

TARGET CNS CELL
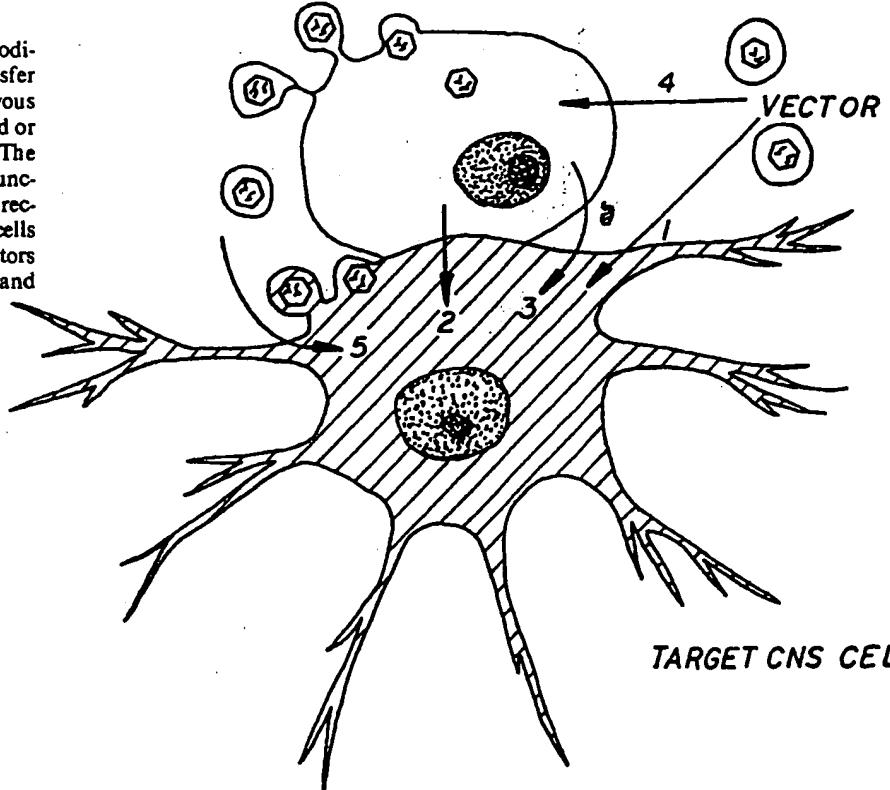
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THERAPY OF CENTRAL NERVOUS SYSTEM BY GENETICALLY MODIFIED CELLS

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5   Department of Health and Human Services. The Government has certain rights in this invention.

Cross-Reference to Related Applications

10   This application is a continuation-in-part of copending patent application United States Serial Number 285,196, filed December 15, 1988, the entire disclosure of which is expressly incorporated by reference herein.

15   ## Technical Field of the Invention

The present invention relates to the use of recombinant technology for genetic modification of donor cells for grafting into the central nervous system (CNS) of a subject to

20   treat defects, disease or damage of the CNS. More specifically, the invention relates to the insertion of genes into donor cells, encoding molecules having ameliorative effects on cells in the CNS, including neurons into donor cells such that when the donor cells are grafted into the CNS

25

the molecules are expr ssed and ex rt their ffects on diseased or damaged cells.

Background of The Invention

Attempts to repair the mammalian brain r replace CNS
functions resulting from defects or following disease or
5   damage to the CNS are hampered by an incomplete understanding
of the complex structure-function relationships in the CNS.
Although knowledge of some basic principles of cell function
in the brain has advanced greatly in recent years,
understanding of interactions between clusters of cells or
10   systems and cell circuits in different regions of the brain
and their relationship to the outward manifestations of
behavior and neurological function lags far behind.
Difficulties in approaching these problems have been caused,
in part, by the large number of different cell types in the
15   mammalian CNS and the number and complexity of their
connections. In addition, the blood-brain barrier makes
access to the brain for diagnosis, treatment and the design of
new therapies more difficult.

20   In spite of the absence of sophisticated knowledge of the
pathophysiology of most normal or abnormal brain functions,
some attempts at pharmacological therapy for CNS dysfunction
have already become useful and effective. These include the
use of psycho-active drugs for psychiatric disorders such as
25   schizophrenia, and specific replacement therapy in the rare
cases in which the biochemical and cellular bases of the CNS
disorder are relatively better understood, as in Parkinson's
disease. At the core of most therapeutic approaches is the
objective of replacing or reactivating a specific chemical
30   function in the brain that has been lost as a consequence of
disease or damage.

Intracerebral neural grafting has emerged recently as an
additional potential approach to CNS therapy. The replacement
35   or addition of cells to the CNS which are able to produce and
secrete therap utically useful metabolites may ffer the
advantage of averting repeated drug administration while also

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avoiding the drug delivery complications posed by the
blood-brain barrier. (Ros nst in, _Science_ 235:772-774 (1987)).
While the concepts and basic procedures of intracerebral
grafting have been known for decades, most of the factors that

5   optimize the survival of grafted cells have only recently come
to be investigated and partially understood. (Bjorklund et
al., in _Neural Grafting in the Mammalian CNS_, p. 709,
Elsevier, Amsterdam (1985); Sladek et al., in _Neural
Transplants: Development and Function_, Plenum Press, NY

10   (1984)). Several factors critical for reliable and effective
graft survival have been identified, including the following:

(1) Age of the donor: efficiency of grafting is reduced
with increasing age of donor cells.

15

(2) Age of the host: young recipients accept grafts
more readily than older ones.

(3) Availability of neurotrophic factors in the host and

20   donor tissue: wound-induced neurotrophic factors enhance
graft survival.

(4) Immunological response: the brain is not totally an
immunologically privileged site.

25

(5) The importance of target-donor matching: neurons
survive better when they are grafted to a site which they
would normally innervate.

30

(6) Vascularization: it is critical that the grafts be
vascularized rapidly or otherwise sufficiently well nourished
from the environment.

35   As these critical factors have become recognized and
optimized, intrac rebral grafting has becom a valid and
r liable t 1 for neurobiologists in th study f CNS function

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and potentially for clinicians for the design f therapies of
CNS disease. This approach has reach d a level f
xperimental clinical application in Parkinson's disease.

5   Parkinson's disease is an age-related disorder
characterized by a loss of dopamine neurons in the substantia
nigra of the midbrain, which have the basal ganglia as their
major target organ. The symptoms include tremor, rigidity and
ataxia. The disease is progressive but can be treated by
10   replacement of dopamine through the administration of
pharmacological doses of the precursor for dopamine, L-dopa,
(Marsden, Trends Neurosci. 9:512 (1986); Vinken et al., in
Handbook of Clinical Neurology p. 185, Elsevier, Amsterdam
(1986)), although with chronic use of pharmacotherapy the
15   patients often become refractory to the continued effect of
L-dopa. There are many suggested mechanisms for the
development of the refractory state, but the simplest is that
the patients reach a threshold of cell loss, wherein the
remaining cells cannot synthesize sufficient dopamine from the
20   precursor.

        Parkinson's disease is the first disease of the brain for
which therapeutic intracerebral grafting has keen used in
humans. Several attempts have been made to provide the
25   neurotransmitter dopamine to cells of the diseased basal
ganglia of Parkinson's patients by homografting adrenal
medullary cells to the brain of affected patients. (Backlund
et al., J. Neurosurg. 62:169-173 (1985); Madrazo et al., New
Eng. J. Med. 316:831-836 (1987)). The transplantation of
30   other donor cells such as fetal brain cells from the
substantia nigra, an area of the brain rich in dopamine-
containing cell bodies and also the area of the brain most
affected in Parkinson's disease, has been shown to be
effective in reversing the behavioral deficits induced by
35   selective dopaminergic neurotoxins. (Bjorklund et al., Ann.
N.Y. Acad. Sci. 457:53-81 (1986); Dunnett et al., Trends
Neurosci. 6:266-270 (1983)). These experiments suggest that

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synaptic connectivity may not be a requisite for a functi nal graft and that it may be sufficient to have cells constitutively producing and secr ting dopamine in the vicinity of the defective cells.

5

With this background, it seems likely that Parkinson's disease is a candidate disease for the transplantation of genetically engineered cells, because (1) the chemical deficit is well known (dopamine), (2) the human and rat genes for the

10   rate-limiting enzyme in the production of dopamine have been cloned (tyrosine hydroxylase), (3) the anatomical localization of the affected region has been identified (basal ganglia), and (4) synaptic connectivity does not appear to be required for complete functional restoration.

15

The recent demonstration of genetic components in a rapidly growing list of other CNS diseases, including Huntington's disease, (Gusella et al., Nature 306:234-238 (1983)) Alzheimer's disease, (Delabar et al., Science, N.Y.

20   235, 1390-1392 (1987); Goldgaber et al., Science, N.Y. 235:877-880 (1987); St. George-Hyslop et al., Science, N.Y. 235:885-890 (1987); Tanzi et al., Science, N.Y. 235:880-884 (1987)); bipolar disease (Baron et al., Nature 326:289-292 (1987)); schizophrenia (Sherrington et al., Nature 336:164-167

25   (1988) and many other major human diseases, suggests that gene therapy is an useful approach to treat these CNS diseases.

In parallel to the progress in neurobiology during the past several decades, advances in an understanding of

30   molecular biology and the development of sophisticated molecular genetic tools have provided new insights into human disease in general. As a result, medical scientists and geneticists have developed a profound understanding of many human diseases at the biochemical and genetic levels. The

35   normal and abnormal biochemical features of many human genetic diseases have become understood, the relevant genes have b en isolated and characterized, and early model systems have been

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devel ped for the introduction f functional wild-type g nes
int mutant c lls t c rr ct a dis ase ph notype. (And rs n,
Science 226:401-409 (1984)). The extensi n f this approach
to whole animals, that is, the correction of a disease

5   phenotype _in vivo_ through the use of the functional gene as a
pharmacologic agent, has come to be called "gene therapy".
(Friedmann et al., _Science_ 175:949-955 (1972); Friedmann, _Gene
Therapy Fact and Fiction_, Cold Spring Harbor Laboratory, New
York (1983)). Gene therapy is based on the assumption that the

10   correction of a disease phenotype can be accomplished either
by modification of the expression of a resident mutant gene or
the introduction of new genetic information into defective or
damaged cells or organs _in vivo_.

15   At present, techniques for the ideal versions of gene
therapy, that is through site-specific gene sequence
correction or replacement _in vivo_ are just beginning to be
conceived but are not yet well developed. Therefore, most
present models of gene therapy are actually genetic

20   augmentation rather than replacement models and rely on the
development of efficient gene-transfer systems to introduce
functional, wild-type genetic information into genetically
defective cells _in vitro_ and _in vivo_. To be clinically
useful, the availability of efficient delivery vectors for

25   functional DNA sequences (transgenes) must be combined with
easy accessibility of suitable disease-related target cells or
organs and with the development of techniques to introduce the
vector stably and safely into those target cells.

30   Model systems for the genetic and phenotypic correction
of simple enzymatic deficits are now being developed and
studied, as is the identification of the appropriate potential
recipient cells and organs associated with specific metabolic
and genetic diseases. Evidence has recently been obtained to

35   show that foreign genes introduced into fertilized mouse eggs
can c rrect disease ph n typ . (C nstantini et al., _Science_
233:1192-1394 (1986); Mason t al., _Science_ 234:1372-1378

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(1986); and Readhead t al., Cell 48:703-712 (1987)).

A great deal of attention has recently been paid t the
use of gene delivery vectors derived from murine retroviruses

5   (Anderson, Science 226:401-409 (1984); Gilboa et al,
Biotechniques 4:504-512 (1986)) for gene transfer. Gene
transfer in vitro using such retroviral vectors is extremely
efficient for a broad range of recipient cells, the vectors
have a suitably large capacity for added genes, and infection

10   with them does little metabolic or genetic damage to recipient
cells. (Shimotohno et al., Cell 26:67-77 (1981); Wei et al.,
J. Virol . 39:935-944 (1981); Tabin et al., Molec. Cell. Biol.
2:426-436 (1982)). Several useful systems have demonstrated
that the expression of genes introduced into cells by means of

15   retroviral vectors can correct metabolic aberrations in vitro
in several human genetic diseases associated with single-gene
enzyme deficiencies. (Willis et al., J. Biol. Chem.
259:7842-7849 (1984); Kantoff et al., Proc. Natl. Acad. Sci.
USA 83:6563-6567 (1986)). There has been particular interest

20   in bone marrow as a potential target organ for this approach
to gene therapy because of the prevalence and importance of
disorders of bone marrow-derived cell lineages in a variety of
major human diseases, including the thalassemias and
sickle-cell anemia, Gaucher's disease, chronic granulomatous

25   disease (CGD) and immunodeficiency disease resulting from
deficiencies of the purine pathway enzymes, adenosine
deaminase (ADA) and purine nucleoside phosphorylase (PNP)
(Kantoff, supra; McIvor et al., Molec. Cell. Biol. 7:838-846
(1987); Soriano et al., Science 234:1409-1413 (1986); Willis

30   et al., supra)). Other metabolically important target organs,
such as the liver, have also recently become theoretically
susceptible for genetic manipulation through the demonstration
of infection of cells from such organs with viral vectors
(Wolff et al., Proc. Natl. Acad. Sci. USA 84:3344-3348

35   (1987)). Furthermore, the discovery of numerous cell-specific
r gulatory signals such as cis-acting enhancers,
tissue-specific promoters and oth r sequences may pr vide

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tissue specific gene expression in many ther organs even
aft r general, non-specific infections and g ne transfer in
vivo (Khoury et al., Cell 33:313-314 (1983); Serflin t al.,
Trends Genet. 1:224-230 (1985)).

5

A recently developed model of gene therapy uses target
cells removed from a subject, placed in culture, genetically
modified in vitro, and then re-implanted into the subject
(Wolff et al., Rheumatic Dis. Clin. N. Amer. 14(2) 459-477
10   (1988); Eglitis et al. Biotechniques 6:608-614 (1988)).
Target cells have included bone marrow stem cells (Joyner et
al., Nature 305:556-558 (1983); Miller et al., Science
225:630-632 (1984); Williams et al., Nature 310:476-480
(1984)); fibroblasts (Selden et al., Science 236:714-718
15   (1982); Garver et al., Proc. Natl . Acad. Sci. USA
84:1050-1054 (1987) and St. Louis et al., Proc. Natl. Acad.
Sci. USA 85:3150-3154 (1988)), keratinocytes (Morgan et al.,
Science 237:1476-1479 (1987)) and hepatocytes (Wolff et al.,
Proc. Natl. Acad. Sci. USA 84:3344-3348 (1987)). This
20   indirect approach of in vivo gene transfer is necessitated by
the inability to transfer genes efficiently directly into
cells in vivo. Although there has been some recent progress
towards genetically modifying neurons in culture (Geller
et al., Science 241:1667-1669 (1988)), this indirect approach
25   of in vivo gene transfer has not yet been applied to the CNS.

There are several ways to introduce a new function into
target cells in the CNS in a phenotypically useful way i.e. to
treat defects, disease or dysfunction (Fig. 1). The most
30   direct approach, which bypasses the need for cellular grafting
entirely, is the introduction of a transgene directly into the
cells in which that function is aberrant as a consequence of a
developmental or genetic defect, i.e. neuronal cells in the
case of Tay-Sachs disease, possibly Lesch-Nyhan disease, and
35   Parkinson's disease (1, in Fig. 1). Alternatively, a new
function is expressed in defectiv target c lls by introducing
a genetically m dified donor c ll that c uld establish gap

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junction r other contacts with the target c ll (2, in Fig.
1). Some such contacts are known t permit the efficient
diffusi n f m tabolically important small mol cules fr m on
cell to another, leading to phenotypic changes in the

5   recipient cell (Lowenstein, *Biochim. Biophys. Acta*. 560:1-66
(1979)). This process has been called "metabolic cooperation"
and is known to occur between fibroblasts and glial cells
(Gruber et al., *Proc. Natl. Acad. Sci. USA* 82:6662-6666
(1985)), although it has not yet been demonstrated

10   conclusively in neurons. Still other donor cells could
express and secrete a diffusible gene product that can be
taken up and used by nearby defective target cells (3, in Fig.
1). The donor cells may be genetically modified *in vitro* or
alternatively they may be directly infected *in vivo* (4, in

15   Fig. 1). This type of "co-operativity" has been demonstrated
with CNS cells, as in the case of NGF-mediated protection of
cholinergic neuronal death following CNS damage (Hefti, *J.*
Neurosci. 6:2155 (1986); Williams et al., *Proc. Natl. Acad.*
Sci. USA 83:9231-9235 (1986)). Finally, an introduced donor

20   cell infected with not only replication-defective vector but
also replication-competent helper virus, could produce locally
high titers of progeny virus that might in turn infect nearby
target cells to provide a functional new transgene (5, in Fig.
1).

25

There are several types of neurons in the mammalian
brain. Cholinergic neurons are found within the mammalian
brain and project from the medial septum and vertical limb of
the diagonal band of Broca to the hippocampal formation in the

30   basal forebrain. The short, nerve-like portion of the brain
connecting the medial septum and vertical limb of the diagonal
band with the hippocampal formation is termed the "fimbria
fornix". The fimbria fornix contains the axons of the neurons
located in the medial septum and diagonal band. An accepted

35   model of neuron survival *in vivo* is the survival of septal
ch linergic neur ns after fimbria fornix trans cti n r lesion
(als termed "axotomy"). Axotomy severs th ch linergic

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neurons in the septum and diagonal band and results in the
death of up t   n -half f th ch linergic n ur ns (Gage t
al., Neuroscience 19:241-256 (1986)). This degenerativ
response is attributed to the loss of trophic support from

5   nerve growth factor (NGF), which is normally transported
retrogradely in the intact brain from the hippocampus to the
septal cholinergic cell bodies (Korsching et al., Proc. Natl.
Acad. Sci. USA 80:3513 (1983); Whittemore et al., Proc. Natl.
Acad. Sci. USA 83:817 (1986); Shelton et al., Proc. Natl.

10   Acad. Sci. USA 83:2714 (1986); Larkfors et al., J. Neurosci.
Res. 18:525 (1987); and Seilor et al., Brain Res. 300:33
(1984)).

Studies have shown that chronic intra-ventricular
15   administration of NGF before axotomy will prevent cholinergic
neuron death in the septum (Hefti, J. Neurosci. 8:2155-2162
(1986); Williams et al., Proc. Natl. Acad. Sci. USA 83:9231
(1986); Kromer, Science 235:214 (1987); Gage et al. J. Comp.
Neurol. 269:147 (1988)). Axotomy thus provides an in vivo

20   model for determining at various points in time the ability of
various therapies to prevent retrograde neuronal death.

One of the characteristics of the adult mammalian CNS is
that new axons generated following perturbation can only grow

25   a relatively short distance within the brain (Cajal, in
Degeneration and Regeneration of the Nervous System, Oxford
University Press, London, England, (1928)). This inability of
adult neurons to regenerate in response to damage may be due
to the following: 1) Activation of astrocytes, which are

30   support and nutritive cells found throughout the CNS,
following injury results in the formation of scar tissue which
acts as a physical barrier to regenerating axons such that
fibers are unable to traverse the scar tissue; thus astrocytic
scars are unable to provide a conducive substrate for axon

35   elongation (Cajal, supra; Brown, J. Comp. Neurol. 87:131
(1947); Clemente, in Regen rati n in the Central Nerv us
System, ed. Windle, pp. 147-161, Thomas, Springfield, (1955);

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Windle, Physiol. Rev. 36:426 (1956); and R ier et al., in Spinal C rd Reconstructi n, ds. Kao et al., pp. 163-195, Raven, New York, (1983)). Astrocytes in th immature CNS, n the other hand, play an important role in the guidance of

5   elongating axons (Silver et al., J. Comp. Neurol. 210:10-29 (1982)); 2) Myelin-associated substances released following damage to the brain have been shown to inhibit axon growth in vitro (Schwab and Croni, J. Neurosci. 8:2381-2393 (1988)) ; 3) The lack of axonal regeneration in the adult CNS may be due,

10   in part, to inadequate levels of trophic or tropic molecules which induce neuronal regeneration and promote axon growth, respectively (Reier et al., in Neural Regeneration and Transplantation, pp. 183-209, Alan R. Liss, New York, (1989) and Schwartz et al., FASEB J. 3:2371-2378 (1989)).

15

      Despite many factors which may impede axon regrowth within the adult brain, certain neurons in the rat CNS, especially retinal ganglion neurons and septal cholinergic neurons, exhibit a remarkable potential to extend new axons

20   into substrates of various types, so-called "neural bridges", including segments of autologous peripheral nerve (David and Aguayo, Science 214:931 (1981); Benfry and Aguayo, Nature 296:150 (1982); Wendt et al., Exp. Neurol. 79:452 (1983); and Hagg et al., Exp. Neurol. 109:153 (1990)), cultured Schwann

25   cells within a collagen matrix (Kromer and Cornbrooks, Proc. Natl. Acad. Sci. USA 82:6330 (1985)), embryonic rat hippocampus (Kromer et al., Brain Res. 210:153 (1981)), and human amnionic membrane (Gage et al., Exp. Brain Res. 72:371 (1988). Connectivity between the septum and hippocampus of

30   the brain has also been demonstrated using implants of peripheral homogenates of neurons (Wendt, Brain Res. Bull. 15:13-18 (1985)).

      It would be advantageous, therefore, to develop

35   procedures for gene transfer via efficient vectors followed by intracerebral grafting f the genetically modified cells in vivo so as t am li rat nerve cell dis as , def ct or

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dysfunction, and to promote axonal regeneration, t treat
dis rders of th CNS, such as Alzheimer's disease, Parkins n's
diseas and Huntingt n's disease.

Summary of the Invention

The present invention provides methods for treating
defective, diseased or damaged cells in the mammalian central
nervous system by grafting genetically modified donor cells
into the central nervous system to produce functional
molecules in a sufficient amount to ameliorate the defective,
diseased or damaged cells in the central nervous system. The
cells may be modified using viral or retroviral vectors,
including pLN.8RNL and pLThRNL, containing an inserted
therapeutic transgene or transgenes encoding a product or
products which directly or indirectly affect the cells, or by
other methods of introducing functional DNA into a cell. The
cells may be cultured and injected in suspension into the
central nervous system and may be co-administered with a
therapeutic agent for treating defective, diseased or damaged
cells in the central nervous system. The methods of the
invention include selection, preparation and transfection of
donor cells, and grafting.

The donor cells may be a mixture of cell types from
different anatomical regions and may be primary or
immortalized cells.

Grafting of the donor cells may be accomplished by
multiple grafting of the donor cells into several different
sites within the mammalian CNS. For multiple grafting the
cells may be a mixture of different types of donor cells, or
may be the same or different types of cells containing the
same or different transgenes.

Grafting may be accompanied by implanting th cells in a
substrate mat rial such as a c llagen matrix t prom te

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transplant survival and/or by the use of such material to
facilitate r connecti n between or ameliorative interactions
of injured n ur ns.

5　　　　　Vectors for use in the invention include those carrying a
promoter such as a collagen promoter to enhance expression of
the transgene or transgenes. The vectors may also carry
enhancer sequences to increase the activity of the promoter.

10　　　　The secretion of the functional molecules may be
regulated using a precursor for said molecules. A method of
the invention is the grafting of fibroblasts modified to
express acetylcholine into the CNS and the administration of
choline chloride orally to maintain and enhance the secretion
15　　of acetylcholine from the grafted fibroblasts.

The invention includes a method for enhancing the
expression of a therapeutic transgene product from quiescent
donor cells grafted into a mammalian central nervous system,
20　　by inserting a quiescent promoter into a vector used to
genetically modify the donor cells. The promoter may be a
non-viral promoter such as a collagen promoter. Additional
enhancer sequences may be inserted with the promoter.
Cytokines may be administered to regulate promoter activity to
25　　enhance transgene expression.

Immunosuppression agents may be used to reduce the
production of Interferon-γ to enhance transgene expression.
Alternatively, an anti-inflammatory agent may be used to
30　　reduce the production of cytokines to enhance the expression
of therapeutic transgenes. Growth factors may also be used to
maintain the survival of the grafted donor cells in the CNS.

Brief Description of the Drawings

35

Fig. 1 is a diagrammatic repr sentation of m th ds for
introducing and analyzing the effect of a new function in

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target cells.

Fig. 2 is a diagrammatic representation of strategies f r
introducing a new function into target cells in the CNS using
5   genetically modified donor cells.

10   Fig. 3 is a diagrammatic depiction of the preparation of
transmissible retrovirus vectors containing a transgene.
(GAG=group specific antigen; Env=envelope; POL=reverse
transcriptase).

15   Fig. 4 is a diagrammatic representation of the linear
restriction maps of the integrated vectors LS P LM and pLNHL$_2$
as described in Example I, infra (arrows indicate the location
of the promoter and the direction of transcription. The
diagonally hatched box of IS P LM represents the human HPRT
20   cDNA encoding a protein with a novel terminal hexapeptide
added by in vitro mutagenesis, LTR=long terminal repeat).

Fig. 5 is a depiction of the circular restriction map of
vector pLNHL$_2$ as described in Example I, infra.
25

Fig. 6a-Fig. 6f are photomicrographs of primary rat
fibroblasts previously infected with hypoxanthine guanine
phosphoribosyl transferase (HPRT) that have been implanted in
rat basal ganglia as described in Example I, infra (Fig. 6a,
30   Fig. 6d = anti- fibronectin; Fig. 6b, Fig. 6e = cresyl violet;
Fig. 6c, Fig. 6f = GFAP; magnification: Fig. 6a-Fig. 6c = 88X;
Fig. 6d-Fig. 6f = 440X).

Fig. 7 is photographs of isoelectric focusing gels for HPRT
35   enzymatic activity of brain extracts from basal ganglia as
described in Example I, infra.

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Fig. 8 is a depiction of the circular restriction map of vect r pLLRNL as d scribed in Example II, _infra_.

Fig. 9 is a depiction of the circular restriction map of
5   vector pPR1 as described in Example II, _infra_.

Fig. 10 is a depiction of the circular restriction map of vector pUCRH as described in Example II, _infra_.
10

Fig. 11 is a diagrammatic depiction of the linear restriction map of the integrated NGF retroviral vector PLN.8RNL containing the 777 base pair Hgal-Pstl fragment of mouse nerve growth factor (NGF) cDNA under control of the viral 5' long
15   terminal repeat (LTR) as described in Example II, _infra_ (arrows indicate transcription initiation sites; psi (𝚿) =retroviral packaging signal; RSV = Rous sarcoma virus promoter; neo^k= neomycin-resistance gene marker.)

20   Fig. 12 is a depiction of the circular restriction map of vector pLN.8RNL as shown in Figure 11 and described in Example II, _infra_.

Fig. 13a - Fig. 13f are photomicrographs of
25   immunohistochemical staining for fibronectin and ChAT as described in Example II, _infra_ (Fig. 13a, Fig. 13b = fibronectin staining in fibroblasts grafted into the fimbria fornix cavity; Fig. 13c - Fig. 13f = coronal sections taken through the medial septum of tissue stained for ChAT; Fig.
30   13a, Fig. 13c, Fig. 13e = animal with graft of retrovirus-infected cells; Fig. 13b, Fig. 13d, Fig. 13f = animal with graft of control cells; Magnification (Mag.): Fig. 13a and 13b = 20X; Fig. 13c and Fig. 13d = 70 X; Fig. 13e and Fig. 13f = 220X).
35

Fig. 14 is a graph sh wing survival of ChAT-immun reactive cells in th septum f a rat in th presence and absence f

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NGF as d scribed in Example II, _infra_.

Fig. 15a - Fig. 15f are phot micrographs of
acetylcholinesterase histochemistry as described in Example

5   II, _infra_ (Fig. 15a = low power magnification of an animal
grafted with NGF-infected donor cells; Fig. 15c = higher power
magnification of Fig. 15a through the medial septum; Fig. 15e
= high power magnification of Fig. 15a through the dorsal
lateral quadrant of the septum; Fig. 15b, Fig. 15d, Fig. 15f =

10   animal grafted with control cells as described for Fig. 15a,
Fig. 15c, Fig. 15e; Mag.: Fig. 15a, Fig. 15b = 20X; Fig. 15c -
Fig. 15f = 220X).

Fig. 16 is a diagrammatic depiction of the linear restriction

15   map of pLThRNL retroviral integrated vector as described in
Example III, _infra_ (arrows indicate the location of the
promoters and the direction of transcription; LTR = long
terminal repeat; RSV = modified RSV promoter; neo^R =
neomycin-resistance gene).

20

Fig. 17 is a depiction of the derivation of vector pLThRNL as
shown in Fig. 16 and described in Example II, _infra_.

Fig. 18a-Fig. 18d are photomicrographs of fibroblast grafts to

25   the caudate showing fibronectin immunoreactivity as described
in Example III, _infra_ (Mag. Fig. 18a, Fig. 18c = 10X; Fig.
18b, Fig. 18d = 20X).

Fig. 19 is a graph showing the average percent change in the

30   number of rotations from baseline to post-transplantation in 4
experimental groups of animals as described in Example III,
infra (Top of figure: placement of control and TH-infected
grafts in caudal striatum; Bottom: placement of control and
TH-infected grafts in rostral striatum; bars indicate standard

35   deviation).

Fig. 20 is a graft f gr wth curves f primary skin

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fibroblasts and Rat-1 fibroblasts _in vitro_ as described in Example IV, _infra_.

Fig. 21 are histograms showing the volume (mm^3) of autologous fibroblast grafts after one and four passages in culture prior to grafting and after three and eight weeks post-operative survival, as described in Example IV, _infra_.

Fig. 22 are histograms showing the volume (mm^3) of autologous fibroblast grafts resulting from implantations of 10^4, 2.5 X 10^4, 5 X 10^4 and 10^5 cells/μl as described in Example IV, _infra_.

Fig. 23a - Fig. 23d are photographs showing primary fibroblast grafts (G) in the striatum stained immunohistochemically for fibronectin at three (Fig. 23a, Fig. 23c) and eight (Fig. 23b, Fig. 23d) weeks after implantation as described in Example IV, _infra_ (Mag. Fig. 23a, Fig. 23b = 12X; Fig. 23c, Fig. 23d = 120X).

Fig. 24a - Fig. 24f are photographs of primary fibroblast grafts (G) in the striatum stained with cresyl violet (Fig. 24a, Fig.24b) and stained immunohistochemically for GFAP (Fig. 24c, Fig. 24d) and OX-42 (Fig. 24e, Fig. 24f), at three weeks (Fig. 24a, Fig. 24c, Fig. 24e) and eight weeks (Fig. 24b, Fig. 24d, Fig. 24f) as described in Example IV, _infra_ (Mag. = 120X).

Fig. 25a - Fig.25c are photographs showing the ultrastructure of autologous grafts at eight weeks post implantation (F = fibroblast cell bodies; solid stars = fibroblast processes), as described in Example IV, _infra_ (Mag. Fig. 25a = 6,600X; Fig. 25b = 9,100X; Fig. 25c = 16,600X).

Fig. 26a - Fig.26b are photographs showing astrocytic pr cesses and phagocytic cells present in th grafts (As = astrocytic pr c sses) as d scribed in Example IV, _infra_ (Mag.

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Fig. 26a = 16,600X; Fig. 26b = 15,000X).

Fig. 27a - Fig. 27d are photographs showing graft vasculature
as described in Example IV, infra (Fig. 27a, E = non-
5   fenestrated endothelial cells; Fig. 27b, arrowheads =
invaginations; Fig. 27c, arrows = intercellular junctions;
Fig. 27d, As = astrocytic processes; Mag. Fig. 27a= 13,200X;
Fig. 27b=25,400X; Fig. 27c=25,000X; Fig. 27d=16,600X).

10   Fig. 28 is a photograph showing the results of in vitro
labelling for tyrosine hydroxylase of primary fibroblasts
containing a TH transgene as described in Example V, infra
(Mag. = 25X).

15   Fig. 29 are graphs showing the results of grafts on
apomorphine-induced rotational behavior of 6-OHDA-lesioned
rats as described in Example V, infra (Control, top; βGal
fibroblast grafts, FF2/βGal, middle; TH fibroblasts, FF2/TH,
bottom; PRE = prior to grafting, ** = $p < 0.01$, * = $p < 0.05$).
20

Fig. 30a - Fig. 30b are photomicrographs showing the results
of in situ hybridization to TH primary fibroblasts grafted
into the striatum of rats as described in Example V, infra
(Fig. 30a graft of FF2/TH cells; 30b graft of FF2/βGal cells
25   (control); G = graft; V = ventricle)).

Fig. 31a - Fig. 31d are photographs showing the results of in
vivo labelling of grafted cells for either βGal histochemistry
or in situ hybridization to TH mRNA as described in Example V,
30   infra (Fig. 31a FF2/βGal graft stained for βGal
histochemistry; Fig. 31b higher magnification of FF2/βGal
graft; Fig. 31c in situ hybridization for TH mRNA in FF2/TH
graft, arrow = fibroblasts; Fig. 31d in situ hybridization to
TH mRNA in FF2/βGal graft; Mag. Fig. 31a = 10X; Fig. 31b, Fig.
35   31c and Fig. 31d = 40X).

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Fig. 32a- Fig. 32b are photographs showing the TH-
immunoreactivity of FF2/TH fibroblasts _in vivo_ as described in
Example V, _infra_ (asterisks = rhodamine fluorescence; Mag. =
20X).

5

Fig. 33 is a graph depicting the secretion of human NGF from
genes and cell types as indicated, as described in Example VI,
infra.

10   Fig. 34 is a schematic representation of the pLChRNL vector as
described in Example VII, _infra_.

Fig. 35a- Fig. 35f are photographs of immunostained
fibroblasts as described in Example VII, _infra_.

15

Fig. 36a - Fig. 36c illustrate the results of: Northern blot
analysis of total RNA from fibroblasts (36a); assay of ChAT
activity in fibroblasts (36b) and HPLC analysis of ACh
secreted into the medium by fibroblasts (36c), as described in
20   Example VII, _infra_.

Fig. 37a - Fig. 37b are graphs depicting the results of adding
choline chloride to cultures of Rat-1/dChAT cells as
determined by HPLC and described in Example VII, _infra_ (Fig.
25   37a = effects on intracellular and extracellular ACh; Fig. 37b
= effects on ChAT activity).

Fig. 38a - Fig. 38c illustrate the results of quiescence
induced by serum starvation on the expression of dChAT as
30   shown by the effects on: ChAT activity (Fig. 38a); steady
state levels of dChAT mRNA (Fig. 38b) and on the ratio of
dChAT/cyclophilin (cyc.) mRNA (Fig. 38c), as described in
Example VII, _infra_.

35

Fig. 39a - Fig. 39c illustrate the effects f quiescence

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induced by c ntact inhibition on the xpr ssion of dChAT as
shown by the ffects on: ChAT activity (Fig. 39a); levels of
dChAT mRNA (Fig. 39b); and on the ratio of dChAT/cyc. (Fig.
39c), as described in Example VII, infra.

5

Fig. 40 is a bar graph showing the effect of choline on the
release of ACh from confluent fibroblasts as described in
Example VII, infra.

10   Fig. 41 is a depiction of the circular restriction map of
vector pSVS32ACAT, as described in Example VIII, infra.

Fig. 42 is a depiction of the circular restriction map of
vector pMLVCAT, as described in Example VIII, infra.

15
Fig. 43 is a depiction of the circular restriction map of
vector pCMVCAT, as described in Example VIII, infra.

Fig. 44 is a bar graph depicting the relative CAT expression
20   of the SV 40, LTR and Collagen promoters (Coll and Coll(E)
promoter-enhancer) as described in Example VIII, infra.

Fig. 45a - Fig. 45b are photographs of Northern blots showing
the effects of TGFβ, IL-1β and TNFα on dChAT-expressing
25   fibroblasts, as described in Example IX, infra (Fig. 45a:
effect of TGF-β (lane 2) and IL-1β (lane 3); Fig. 45b: effect
of Infγ, lane 1 = control; cyclophilin (Cyc) = internal
control; lane 2 = effects after 24 hrs; lane 3 = effects after
48 hrs; dChAT = dChAT proviral mRNA).

30
Fig. 46 is a photograph of a Northern blot showing the effects
of coadministration of TGFβ and IL-1β, and dexamethasone, on
confluent dChAT-producing fibroblasts, as described in Example
IX, infra (lane 1 = control; lane 2 = TGFβ/IL-1β; lane 3 =
35   dexamethasone alone; lane 4 = TGFβ/IL-1β and dexamethasone;
t p signals = dChAT mRNA; lower signals = cyclophilin mRNA).

Fig. 47a - Fig. 47b ar bar graphs showing the activity of the collagen pr moter in 10% versus 2% serum (Fig. 47a) and the effect of various cytokines on the c llagen promoter (Fig. 47b) as described in Example IX, _infra._

5

Fig. 48a - Fig. 48d are photomicrographs showing grafts of LTR-CAT (Fig. 48a, Fig. 48b) or Coll(E)-CAT (Fig. 48c, Fig. 48d) cells grafted into the striatum of adult Fischer rats as described in Example IX, _infra_ (white arrows = fibroblasts).

10

Fig. 49a - Fig. 49d are photomicrographs showing horizontal sections through the left and right striatum at one (Fig. 49a, Fig. 49b) and eight (Fig. 49c, Fig. 49d) weeks after implants (genetically modified cells injected into the right striatum

15   and non-infected controls injected into the left striatum) as described in Example X, _infra_ (G = grafts; NG = nucleus basalis of Meynert; RT = reticular thalamic nucleus; scale bars: Fig. 49a, Fig. 49b, 0.45 mm; Fig. 49c, Fig. 49d, 0.22 mm).

20

Fig. 50 is an electron photomicrograph of a graft composed of NGF-producing cells eight weeks post-implantation as described in Example X, _infra_ (F = fibroblasts; E = endothelial cells; arrow = astrocytic processes; scale bar = 1.0 μm).

25

Fig. 51 is a photomicrograph and schematic illustration of an axo-glial arrangement within a graft of NGF-producing cells at eight weeks as described in Example X, _infra_ (Ax = unmyelinated axons; As = reactive astrocytic processes;

30   arrowheads = basal lamina; scale bar = 1.0 μm).

Fig. 52a - Fig. 52g are schematic illustrations of a sagittal view of the adult rat forebrain after unilateral fimbria-

35   fornix (FF) ablation and placement of a graft consisting of collagen with either NGF-producing or contr l, n n-infected primary fibroblasts, as described in Example X, _infra_ (MS =

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media septum, G = graft, and DG = hippocampal dentat gyrus,
Fig. 52a, Fig. 52b = coronal s ctions at comparable l vels
through the medial septum stain d immunohist chemically f r
NGF receptor after unilateral FF aspirative lesion and

5   placement of either NGF-producing (Fig. 52a) or control (Fig.
52b) grafts (arrowheads indicate septal midline); Fig. 52c,
Fig. 52d = coronal sections through grafts of either NGF-
producing (Fig. 52c) or control, non-infected (Fig. 52d)
fibroblasts stained for AChE (dotted line represents

10   approximate boundary of the graft, scale bars in Fig. 52b and
52c = 100 μm; scale bar in Fig. 52d = 200 μm); Fig. 52e, Fig.
52f, Fig. 52g = coronal sections through the hippocampal
dentate gyrus stained for AChE (G = granular layer;, IM =
inner molecular layer; OM = outer molecular layer, and P =

15   polymorphic layer)).

Fig. 53a - Fig. 53d are photomicrographs showing retrogradely
fluorescently labeled septal neurons following stereotaxic
placement of fluorescent microspheres in the dentate gyrus

20   after surgery in animals that received NGF-producing grafts as
described in Example X, infra (Fig. 53a = ipsilateral medial
septum; Fig. 53b = contralateral diagonal band; Fig. 53c =
septal midline; and Fig. 53d = ipsilateral diagonal band;
scale bar = 50 μm).

25
Fig. 54a- Fig. 54f are electron photomicrographs showing the
ultrastructural distribution of unmyelinated axons within
grafts of collagen and NGF-producing fibroblasts after surgery
as described in Example X, infra (* = astrocytic processes;

30
S = Schwann cells; L = lumen of the capillary; scale bars =
1.0 μm).

Fig. 55a - Fig. 55f depicts topographical (Fig. 55a) and
35   synaptic (Fig. 55b-f) distribution of AChE activity within the
dentate gyrus after FF ablati n and implants of NGF-producing
fibr blasts in a c llagen matrix as described in Example X,

infra (Fig. 55a = coronal section (40 μm) of the dentat gyrus stained for AChE, tak n immediately adjacent to that tissue examined at the ultrastructural level, IM = inner molecular layer, OM = outer molecular layer; G =granular layer; P = polymorphic layer; Fig. 55b = granular layer; Fig. 55c = molecular layer; Fig. 55 d,e,f, arrowheads = synaptic contacts, Sh = dendritic shafts; Sp = spines, As = astrocytic processes, scale bars: in Fig. 55a = 25 μm; in Fig. 55b = 0.5 μm, in Fig. 55c and representative for Fig. 55d-Fig. 55f = 0.25 μm).

Detailed Description of the Invention

In order that the invention herein described may be more fully understood, the following detailed description is set forth.

The present invention relates to a process for grafting genetically modified donor cells into the mammalian central nervous system (CNS) to treat disease or trauma of the CNS. More particularly, the invention relates to the use of vectors carrying functional gene inserts (transgenes) to modify donor cells to produce molecules that are capable of directly or indirectly affecting cells in the CNS to repair damage sustained by the cells from defects, disease or trauma. Preferably, for treating defects, disease or damage of cells in the CNS, donor cells such as fibroblasts are modified by introduction of a retroviral vector containing a transgene or transgenes, for example a gene encoding nerve growth factor (NGF) protein. The genetically modified fibroblasts are grafted into the central nervous system, for example the brain, to treat defects, disease such as Alzheimer's or Parkinson's, or injury from physical trauma, by restoration or recovery of function in the injured neurons as a result of production of the expressed transgene product(s) from the genetically modified donor cells. The donor cells may also be used to introduc a transgene product or products int the CNS

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that enhance the production of endogen us m l cules that have
am liorativ effects _in vivo_.

5   As used herein the term "transgene" or "therapeutic
transgene" means DNA inserted into a donor cell encoding an
amino acid sequence corresponding to a functional protein
capable of exerting a therapeutic effect on cells of the CNS
or having a regulatory effect on the expression of a function
in the cells of the CNS.

10

                    Gene Transfer Into Donor Cells In Vitro

        The strategy for transferring genes into donor cells _in
vitro_ is outlined in Fig. 2 and includes the following basic
15   steps: (1) selection of an appropriate transgene or transgenes
whose expression is correlated with CNS disease or
dysfunction; (2) selection and development of suitable and
efficient vectors for gene transfer; (3) preparation of donor
cells from primary cultures or from established cell lines;
20   (4) demonstration that the donor implanted cells expressing
the new function are viable and can express the transgene
product(s) stably and efficiently; (5) demonstration that the
transplantation causes no serious deleterious effects; and (6)
demonstration of a desired phenotypic effect in the host
25   animal.

        The functional molecules produced by transgenes for use
in the invention include, but are not limited to, growth
factors, enzymes, gangliosides, antibiotics,
30   neurotransmitters, neurohormones, toxins, neurite promoting
molecules, antimetabolites and precursors of these molecules.
In particular, transgenes for insertion into donor cells
include, but are not limited to, tyrosine hydroxylase,
tryptophan hydroxylase, NGF, ChAT, GABA-decarboxylase, Dopa
35   decarboxylase (AADC), enkephlin, ciliary neuronal trophic
fact r (CNTF), brain derived neur trophic factor (BDNF),
neurotr phin (NT)-3, NT-4, and basic fibroblast gr wth factor

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(bFGF).

Genetic Modification of Donor Cells

5   The methods described below to modify donor cells using
retroviral vectors and grafting into the CNS are merely for
purposes of illustration and are typical of those that might
be used. However, other procedures may also be employed, as
is understood in the art.

10
          Most of the techniques used to transform cells, construct
vectors and the like are widely practiced in the art, and most
practitioners are familiar with the standard resource
materials which describe specific conditions and procedures.
15   However, for convenience, the following paragraphs may serve
as a guideline.

Choice of donor cells

          The choice of donor cells for implantation depends
20   heavily on the nature of the expressed gene, characteristics
of the vector and the desired phenotypic result. Because
retroviral vectors require cell division and DNA synthesis for
efficient infection, integration and gene expression (Weiss et
al., RNA Tumor viruses, 2nd Ed., Weiss et al., eds., Cold
25   Spring Harbor Press, New York (1985)), if such vectors are
used, the donor cells are preferably actively growing cells
such as primary fibroblast culture or established cell lines,
replicating embryonic neuronal cells or replicating adult
neuronal cells from selected areas such as the olfactory
30   mucosa and possibly developing or reactive glia. Primary
cells, i.e. cells that have been freshly obtained from a
subject, such as fibroblasts, that are not in the transformed
state are preferred for use in the present invention. Other
suitable donor cells include immortalized (transformed cells
35   that continue to divide) fibroblasts, glial cells, adrenal
cells, hippocampal cells, keratinocytes, hepatocyt s,
c nnective tissue cells, ependymal cells, bone marr w cells,

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stem cells, l uk cytes, chromaffin cells and oth r mammalian
cells susc ptible t genetic manipulation and grafting using
the methods f the present inventi n.

5   The application of methods to induce a state of
susceptibility in stationary, non-replicating target cells may
make many other cell types suitable targets for viral
transduction. For instance, methods have been developed that
permit the successful retroviral vector infection of primary
10   cultures of adult rat hepatocytes, ordinarily refractory to
infection with such vectors, and similar methods may be
helpful for a number of other cells (Wolff et al., Proc. Natl.
Acad. Sci. USA 84:3344-3348 (1987)). In addition, the
development of many other kinds of vectors derived from
15   herpes, vaccinia, adenovirus, or other viruses, as well as the
use of efficient non-viral methods for introducing DNA into
donor cells such as electroporation (Toneguzzo et al., Molec.
Cell. Biol. 6:703-706 (1986)), lipofection or direct gene
insertion may be used for gene transfer intc many other cells
20   presently not susceptible to retroviral vector infection.

          Additional characteristics of donor cells which are
relevant to successful grafting include the age of the donor
25   cells. The results presented herein demonstrate that aged
human cells may be used for transfection with transgenes for
grafting.

                    Choice of Vector

30   Although other vectors may be used, preferred vectors
for use in the methods of the present invention are viral,
including retroviral, vectors. The viral vector selected
should meet the following criteria: 1) the vector must be
able to infect the donor cells and thus viral vectors having
35   an appropriate host range must be selected; 2) the transferred
g ne sh uld be capable f persisting and being expressed in a
cell f r an extended period of time without causing c ll death

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for stabl maint nance and expression in the cell; and 3) the
vect r should do little, if any, damage t targ t cells.
Murine retroviral vect rs offer an effici nt, useful, and
presently the best-characterized means of introducing and

5   expressing foreign genes efficiently in mammalian cells. These
vectors have very broad host and cell type ranges, integrate
by reasonably well understood mechanisms into random sites in
the host genome, express genes stably and efficiently, and
under most conditions do not kill or obviously damage their

10   host cells.

General Methods for Vector Construction

Construction of suitable vectors containing the desired
15   therapeutic gene coding and control sequences employs standard
ligation and restriction techniques, which are well understood
in the art (see Maniatis et al., in Molecular Cloning: A
Laboratory Manual, Cold Spring Harbor Laboratory, New York
(1982)). Isolated plasmids, DNA sequences, or synthesized

20
oligonucleotides are cleaved, tailored, and religated in the
form desired.

Site-specific DNA cleavage is performed by treating with
25   the suitable restriction enzyme (or enzymes) under conditions
which are generally understood in the art, and the particulars
of which are specified by the manufacturer of these
commercially available restriction enzymes (See, e.g. New
England Biolabs Product Catalog.) In general, about 1 μg of

30   plasmid or DNA sequences is cleaved by one unit of enzyme in
about 20 μl of buffer solution. Typically, an excess of
restriction enzyme is used to insure complete digestion of the
DNA substrate.

35   Incubation times of about one hour to two hours at about
37°C are w rkable, although variations can be tolerated. After
each incubati n, pr t in is removed by extracti n with

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phen l/chloroform, and may be followed by　th r extracti n,
and the nucleic acid recovered fr m aqu ous fracti ns by
precipitati n with ethanol. If d sired, size separation of
the cleaved fragments may be performed by polyacrylamide gel
5　　or agarose gel electrophoresis using standard techniques. A
general description of size separations is found in Methods in
Enzymology 65:499-560 (1980).

Restriction cleaved fragments may be blunt ended by
10　　treating with the large fragment of E. coli DNA polymerase I
(Klenow) in the presence of the four deoxynucleotide
triphosphates (dNTPs) using incubation times of about 15 to 25
min at 20°C to 25°C in 50 mM Tris (pH 7.6) 50 mM NaCl, 6 mM
MgCl$_2$, 6 mM DTT and 5-10 μM dNTPs. The Klenow fragment fills
15　　in at 5' sticky ends but chews back protruding 3' single
strands, even though the four dNTPs are present. If desired,
selective repair can be performed by supplying only one of the
dNTPs, or with selected dNTPs, within the limitations dictated
by the nature of the sticky ends. After treatment with
20　　Klenow, the mixture is extracted with phenol/chloroform and
ethanol precipitated. Treatment under appropriate conditions
with S1 nuclease or Bal-31 results in hydrolysis of any
single-stranded portion.

25　　Ligations are performed in 15-50 μl volumes under the
following standard conditions and temperatures: 20 mM Tris-Cl
pH 7.5, 10 mM MgCl$_2$, 10 mM DTT, 33 mg/ml BSA, 10 mM-50 mM NaCl,
and either 40 μM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at
15°C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss)
30　　units T4 DNA ligase at 25°C (for "blunt end" ligation).
Intermolecular "sticky end" ligations are usually performed at
33-100 μg/ml total DNA concentrations (5-100 nM total end
concentration). Intermolecular blunt end ligations (usually
employing a 10-30 fold molar excess of linkers) are performed
35　　at 1 μM total ends concentration.

In vect r constructi n empl ying "vector fragm nts", the

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v ct r fragment is commonly treated with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline ph sphatase (CIP) in rder t rem ve th 5' phosphate and prevent religation of the vector. Digestions are conducted at pH 8 in

5   approximately 150 mM Tris, in the presence of Na+ and Mg+2 using about 1 unit of BAP or CIP per mg of vector at 37°C for about one hour. In order to recover the nucleic acid fragments, the preparation is extracted with phenol/chloroform and ethanol precipitated. Alternatively, religation can be

10   prevented in vectors which have been double digested by additional restriction enzyme digestion of the unwanted fragments.

Methods of preparation of retroviral vectors have been

15   described (Yee et al., Cold Spring Harbor Symp. on Quant. Biol. Vol. LI, pp. 1021-1026 (1986); Wolff et al., Proc. Natl. Acad. Sci. USA 84:3344-3348 (1987); Jolly et al., Meth. in Enzymol. 149:10-25 (1987); Miller et al., Mol. Cell. Biol. 5:431-437 (1985); and Miller, et al., Mol. Cell. Biol.

20   6:2895-2902 (1986) and Eglitis et al., Biotechniques 6:608-614 (1988)) and are now in common use in many laboratories. Retroviral vectors contain retroviral long terminal repeats (LTRs) and packaging (psi, Ψ) sequences, as well as plasmid sequences for replication in bacteria and may include other

25   sequences such as the SV40 early promoter and enhancer for potential replication in eukaryotic cells. Much of the rest of the viral genome is removed and replaced with other promoters and genes. Vectors are packaged as RNA in virus particles following transfection of DNA constructs into

30   packaging cell lines. These include psi (Ψ)2 which produce viral particles that can infect rodent cells and ΨAM and PA 12 which produce particles that can infect a broad range of species.

35   In a preferred viral vector the transgene is brought under the control of either the viral LTR prom ter-enhancer signals or f an internal prom ter, and retain d signals

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within the retroviral LTR can still bring about fficient
integration of th vector into the host cell genome. T
prepare transmissible virus (Fig. 3), recombinant DNA
molecules of such defective vectors are transfected into
5   "producer" cell lines that contain a provirus expressing all
of the retroviral functions required for packaging of viral
transcripts into transmissible virus particles, but lacking
the crucial packaging signal for encapsidation of RNA
transcripts of the provirus into mature virus particles.
10   These include the group specific antigen (GAG) and envelope
(ENV) genes which encode caps id proteins and reverse
transcriptase (POL). Because of this deletion, transcripts
from the helper cannot be packaged into viral particles and
the producer cells, therefore, generate only empty virus
15   particles. However, an integrated defective retroviral vector
introduced into the same cell by means of calcium
phosphate-mediated transfection (Graham and Vander Eb, _Virol_.
52:456-467 (1973)) in which the GAG, ENV and POL genes have
been replaced by the transgene (x) with the intact psi
20   sequence, produces transcripts that can be packaged in _trans_
since they do contain the packaging sequence. The cells
contain two provirus sequences integrated into different sites
of the host cell genome. Because RNA transcripts from the
newly introduced provirus contain the packaging sequence they
25   are efficiently encapsidated into virus particles by means of
viral functions produced in _trans_. Ideally, the result is the
production by the cells of infectious particles carrying the
transgene free of replication-competent wild-type helper
virus. In most, but not necessarily all models of gene
30   therapy, the production of helper virus is probably
undesirable since it may lead to spreading infection and
possibly proliferative disease in lymphoid or other tissue in
the host animal.

35   Because herpes viruses are capable of establishing a
latent infecti n and an apparently non-pathogenic relati nship
with some neural cells, herpes bas d vectors, e.g. HSV-1, may

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be used. Similarly, it should be possible to take advantage
of an ventual improved understanding of oth r human and
animal viruses that infect c lls of the CNS efficiently, such
as rabies virus, measles, and other paramyxoviruses and the

5   human immunodeficiency retrovirus (HIV), to develop useful
delivery and expression vectors. In most cases, with the
exception of rabies virus, these viruses are not truly
neurotropic for infection, but rather have a much more general
susceptible host cell range. They seem, rather, to appear to

10   be neurotropic because the metabolic and physiological effects
of infection are most pronounced in cells of the CNS. It is,
therefore, likely that many vectors derived from these viruses
will be similarly promiscuous in their cell range, and that
CNS specificity for expression must be conferred by the use of

15   appropriate cell-specific enhancer, promoter and other
sequences, such as those that regulate the
oligodendroglial-specific expression of JC virus,
glial-specific expression of the proteolipid protein and glial
fibrillary acidic protein (GFAP) genes, and other possible CNS

20   specific functions in the mouse.

Other virus vectors that may be used for gene transfer
into cells for correction of CNS disorders include
retroviruses such as Moloney murine leukemia virus (MoMuLV);

25   papovaviruses such as JC, SV40, polyoma, adenoviruses;
Epstein-Barr Virus (EBV); papilloma viruses, e.g. bovine
papilloma virus type I (BPV); vaccinia and poliovirus and
other human and animal viruses.

30   A possible problem posed by the use of defective viral
vectors is the potential for the eventual emergence or
"rescue" of pathogenic, replication-competent, wild- type
virus by recombination with endogenous virus-like or other
cellular sequences. This possibility can be reduced through

35   the elimination of all viral regulatory sequences not needed
for the infecti n, stabilization or expressi n of the vector.

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In addition to the above-described m thods for inserting
functional DNA transgenes into don r cells other m thods may
be used. For example, non-vector methods include nonviral
physical transfection of DNA into cells; for example,

5   microinjection (DePamphilis et al., _BioTechnique_ 6:662-680
(1988)); electroporation (Toneguzzo et al., _Molec. Cell. Biol_.
6:703-706 (1986), Potter, _Anal. Biochem._ 174::361-373 (1988));
chemically mediated transfection such as calcium phosphate
transfection (Graham and van der EB, _supra_, Chen and Okayama,

10   _Mol. Cell. Biol._ 7:2745-2752 (1987), Chen and Okayama,
BioTechnique, 6:632-638 (1988)) and DEAE-dextran mediated
transfer (McCutchan and Pagano, _J. Natl. Cancer Inst._ 41:351-
357 (1968)); cationic liposomal mediated transfection (Felgner
et al., _Proc. Natl. Acad. Sci. USA_, 84:7413-7417 (1987),

15   Felgner and Holm, _Focus_ 11:21-25 (1989) and Felgner et al.,
Proc. West. Pharmacol. Soc. 32: 115-121 (1989)) and other
methods known in the art.

Mechanisms of Phenotypic Correction by Donor Cells

20

Preparation of Donor Cells

The donor cells must be properly prepared for grafting.
For example, for injection of genetically modified donor cells

25   according to the present invention, cells such as fibroblasts
obtained from skin samples are placed in a suitable culture
medium for growth and maintenance of the cells, for example a
solution containing fetal calf serum (FCS) and allowed to grow
to confluency. The cells are loosened from the culture

30   substrate for example using a buffered solution such as
phosphate buffered saline (PBS) containing 0.05% trypsin and
placed in a buffered solution such as PBS supplemented with
1 mg/ml of glucose; 0.1 mg/ml of $MgCl_2$; 0.1 mg/ml $CaCl_2$
(complete PBS) plus 5% serum to inactivate trypsin. The cells

35   may be washed with PBS using centrifugation and are then
resuspended in the complete PBS with ut trypsin and at a
selected d nsity for injection. In addition t PBS, any

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osmotically balanced soluti n which is physiologically
compatibl with th host subj ct may be used to susp nd and
inj ct the donor c lls int the host.

5   The long-term survival of implanted cells may depend on
effects of the viral infection on the cells, on cellular
damage produced by the culture conditions, on the mechanics of
cell implantation, or the establishment of adequate
vascularization, and on the immune response of the host animal
10   to the foreign cells or to the introduced gene product. The
mammalian brain has traditionally been considered to be an
immunologically privileged organ, but recent work has shown
conclusively that immune responses can be demonstrated to
foreign antigens in the rat brain. It is important to
15   minimize the potential for rejection and graft-versus-host
reaction induced by the grafted cells by using autologous
cells wherever feasible, by the use of vectors that will not
produce changes in cell surface antigens other than those
associated with the phenotypic correction and possibly by the
20   introduction of the cells during a phase of immune tolerance
of the host animal, as in fetal life.

       The most effective mode and timing of grafting of the
transgene donor cells of the invention to treat defects,
25   disease or trauma in the CNS of a patient will depend on the
severity of the defect and on the severity and course of
disease or injury to cells such as neurons in the CNS, the
patient's health and response to treatment and the judgment of
the treating health professional.
30

       Of course, as in all other gene-transfer systems, the
important issues of appropriate or faithful gene expression
must be resolved to ensure that the level of gene expression
is sufficient to achieve the desired phenotypic effect and not
35   so high as to be toxic to the cell.

       A pr blem associated with the use of g n tically

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engineered cells as transplants for gene therapy is that as
cells becom qui scent (n n-dividing) th xpressi n of genes,
including transgenes, has be n observed t d crease ("down
regulate") (Palmer et al., Proc. Natl. Acad. Sci. USA 88:1330-

5   1334 (1991)). Primary fibroblasts grafted into the brain do
not continue to divide when implanted unless they are
transformed and tumorigenic. They thus exist in a quiescent
state in the brain. It is thus useful to provide means for
maintaining and/or increasing expression of the transgene in

10   the absence of cell division to promote long term stable
expression of therapeutic genes used in fibroblasts for gene
therapy.

        Expression of a gene is controlled at the transcription,

15   translation or post-translation levels. Transcription
initiation is an early and critical event in gene expression.
This depends on the promoter and enhancer sequences and is
influenced by specific cellular factors that interact with
these sequences. The transcriptional unit of many prokaryotic

20   genes consists of the promoter and in some cases enhancer or
regulator elements (Banerji et al., Cell 27:299 (1981); Corden
et al., Science 209:1406 (1980); and Breathnach and Chambon,
Ann. Rev. Biochem. 50:349 (1981)). For retroviruses, control
elements involved in the replication of the retroviral genome

25   reside in the long terminal repeat (LTR) (Weiss et al., eds.,
In: The molecular biology of tumor viruses: RNA tumor viruses,
Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
(1982)). Moloney murine leukemia virus (MLV) and Rous sarcoma
virus (RSV) LTRs contain promoter and enhancer sequences

30   (Jolly et al., Nucleic Acids Res. 11:1855 (1983); Capecchi et
al., In: Enhancer and eukaryotic gene expression, Gulzman and
Shenk, eds., pp. 101-102, Cold Spring Harbor Laboratories,
Cold Spring Harbor, New York). Promoter and enhancer regions
of a number of non-viral promoters have also been described

35   (Schmidt et al., Nature 314:285 (1985); Rossi and de
Crombrugghe, Proc. Natl. Acad. Sci. USA 84:5590-5594 (1987)).

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The pr sent invention provides meth ds f r maintaining
and increasing expr ssion f transg n s in quiescent cells
using promoters including c llag n type I (α1 and α2) (Prockop
and Kivirikko, N. Eng. J. Med. 311:376 (1984); Smith and

5   Niles, Biochem. 19:1820 (1980); de Wet et al., J. Biol. Chem.
258:14385 (1983)), SV40 and LTR promoters.

In addition to using viral and non-viral promoters to
drive transgene expression in donor cells such as primary

10   fibroblasts, an enhancer sequence may be used to increase the
level of transgene expression. Enhancers can increase the
transcriptional activity not only of their native gene but
also of some foreign genes (Armelor, Proc. Natl. Acad. Sci.
USA 70:2702 (1973)). For example, in the present invention,

15   collagen enhancer sequences are used with the collagen
promoter α2(I) to increase transgene expression. In addition,
the enhancer element found in SV40 viruses may be used to
increase transgene expression. This enhancer sequence
consists of a 72 basepair repeat as described by Gruss et al.,

20   Proc. Natl. Acad. Sci. USA 78:943 (1981); Benoist and Chambon,
Nature 290:304 (1981), and Fromm and Berg, J. Mol. Appl.
Genetics, 1:457 (1982), all of which are incorporated by
reference herein. This repeat sequence can increase the
transcription of many different viral and cellular genes when

25   it is present in series with various promoters (Moreau et al.,
Nucleic Acids Res. 9:6047 (1891)). In most cases, the SV40
enhancer element is fully functional when it is present in
either orientation and/or at a variety of positions in the
plasmid DNA (Fromm and Berg, supra). The ability of the SV40

30   enhancer to upregulate the transcription from the α2(I)
collagen promoter (Coll) has been previously demonstrated in
embryonic chicken fibroblasts (CEF) and monkey kidney CV-1
cells (Xu et al., In Enhancer and Eukaryotic Gene Expression,
Gulzman and Shenk, eds., Cold Spring Harbor Laboratories, Cold

35   Spring Harbor, New York, pp. 51-54 (1983)). In these cells,
vectors containing the SV40 enhancer region downstream from
th α2(I) collagen promot r increas d chl ramph nical

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acetyltransferase activity, the report r g n used, by 64-fold
and 18-fold in CV-1 and CEF c lls, respectively.

5   Transgene expression may also be increased for long term
stable expression after grafting using cytokines to modulate
promoter activity. Several cytokines have been reported to
modulate the expression of transgene from collagen α2(I) and
LTR promoters (Chua et al., Connective Tissue Res. 25:161-170
(1990); Elias et al., Annals N.Y. Acad. Sci. 580:233-244
10   (1990)); Seliger et al., J. Immunol. 141:2138-2144 (1988) and
Seliger et al., J. Virology 62:619-621 (1988)). For example,
transforming growth factor (TGF)β, interleukin (IL)-1β, and
interferon (Inf)α or γ down regulate the expression of
transgenes driven by various promoters such as LTR. Tumor
15   necrosis factor (TNF)α and TGFβ1 up regulate and IL-1β up
regulates the expression of transgenes driven by the α2(I)
collagen promoter (Coll). The results presented herein
demonstrate that some cytokines such as TNFα, TGFβ and IL-1β,
may be used to control expression of transgenes driven by a
20   promoter in donor cells such as fibroblasts. Other cytokines
that may prove useful include basic fibroblast growth factor
(bFGF) and epidermal growth factor (EGF).

      Collagen promoter with the collagen enhancer sequence
25   (Coll(E)) can also be used to take advantage of the high level
of cytokines present in the brain following grafting of the
modified donor cells to increase transgene expression. In
addition, anti-inflammatory agents including steroids, for
example dexamethasone, may be administered to the graft
30   recipient immediately after implantation of the fibroblasts
and continued, preferably, until the cytokine-mediated
inflammatory response subsides. In certain cases, an
immunosuppression agent such as cyclosporin may be
administered to reduce the production of interferon-γ which
35   downregulates LTR promoter and Coll(E) promoter-enhancer, and
reduces transgene expression.

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For in vivo us , transgenes driven by collag n promoter
ar introduced into c lls and th n directly implanted into the
brain without requiring further intervention. The cytokin s
released after grafting as part of the recipient's natural
5   response will stimulate the collagen promoter driven
transcription of the selected transgene.

Cytokines including the growth factors bFGF and EGF, may
also be administered before, during or after grafting, to
10   promote survival of grafted donor cells in the CNS.

It is also useful to be able to regulate the secretion of
the genetically engineered gene product after grafting. As
shown in an embodiment presented herein, the release of a gene
15   product such as acetylcholine (ACh), a transmitter, greatly
decreased in Alzheimer's Disease, from cultured cells infected
with a MLV vector expressing the choline acetyltransferase
cDNA can be augmented using choline, a precursor for
acetylcholine. This suggests a means for dietary regulation
20   of intracerebral gene therapy.

The genetic correction of some, or many, CNS disorders
may require the establishment or re-establishment of faithful
intercellular synaptic connections. Model systems to study
25   these possibilities have not yet been developed and exploited
because of the paucity of replicating non-transformed
cell-culture systems and the refractoriness of non-replicating
neuronal cells to retroviral infection. However, recent
studies, including those involving the immortalization of
30   embryonic hippocampal neuronal cells, suggest that replicating
neuronal cell culture systems may soon become available for in
vitro gene transfer and then for in vivo implantation
(Caettano and MacKay, Nature 347:762-765 (1990)). Such
neurons might be susceptible to efficient transduction by
35   retroviral or other viral vectors, and if they are also able
t retain th r neur nal characteristics, they may b able to
stablish synaptic c nnections with oth r cells after grafting

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into the brain. Alt rnativ ly, ther are c lls within the CNS
that are late to dev l p, such as th v ntral leaf of th
dentate gyrus of the hippocampus, or continue to divide
through adulthood, such as those in the olfactory mucosa and
5   in the dentate gyrus. Such cells may be suitable targets for
retroviral infection.

The use of non-neuronal cells for grafting may preclude
the development of specific neural connections to resident
10   target cells of the host. Therefore, the phenotypic effects
of fibroblast or other non-neuronal donor cells or target
cells _in vivo_ would be through the diffusion of a required
gene product or metabolite, through gap junctions ("metabolic
co-operation") or through uptake by target cells of secreted
15   donor cell gene products or metabolites. The donor cell may
also act as a toxin "sink" by expressing a new gene product
and metabolizing and clearing a neurotoxin.

Alternatively, neural bridges may be provided which
20   facilitate reconnection between neurons in damaged CNS
tissues. As noted above, grafted donor cells suspended in
substrate material such as collagen matrices can serve as
neural bridges to facilitate axonal regeneration and
reconnection of injured neurons, or may be used in conjunction
25   with neural bridges formed from synthetic or biological
materials, for example homogenates of neurons or placenta, or
neurite promoting extracellular matrices.

Grafting

30

The methods of the invention contemplate intracerebral
grafting of donor cells containing a transgene insert to the
region of the CNS having sustained defect, disease or trauma.

35   Neural transplantation or "grafting" involves trans-
plantati n f cells into th c ntral n rvous system r into
the ventricular caviti s r subdurally ont the surface f a

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host brain.   Conditi ns for successful transplantati n
include: 1) viability of the implant; 2) r t ntion of the
graft at th sit of transplantation; and 3) minimum amount of
pathological reaction at the site of transplantation.

5

Methods for transplanting various nerve tissues, for
example embryonic brain tissue, into host brains have been
described in Neural Grafting in the Mammalian CNS, Bjorklund
and Stenevi, eds., (1985) Das, Ch. 3 pp. 23-30; Freed, Ch. 4,

10   pp. 31-40; Stenevi et al., Ch. 5, pp. 41-50; Brundin et al.,
Ch. 6, pp. 51-60; David et al., Ch. 7, pp. 61-70; Seiger, Ch.
8, pp. 71-77 (1985), incorporated by reference herein. These
procedures include intraparenchymal transplantation, i.e.
within the host brain (as compared to outside the brain or

15   extraparenchymal transplantation) achieved by injection or
deposition of tissue within the host brain so as to be opposed
to the brain parenchyma at the time of transplantation (Das,
supra).

20   The two main procedures for intraparenchymal trans-
plantation are: 1) injecting the donor cells within the host
brain parenchyma or 2) preparing a cavity by surgical means to
expose the host brain parenchyma and then depositing the graft
into the cavity (Das, supra). Both methods provide

25   parenchymal apposition between the graft and host brain tissue
at the time of grafting, and both facilitate anatomical
integration between the graft and host brain tissue. This is
of importance if it is required that the graft become an
integral part of the host brain and to survive for the life of

30   the host.

Alternatively, the graft may be placed in a ventricle,
e.g. a cerebral ventricle or subdurally, i.e. on the surface

35   of the host brain where it is separated from the host brain
par nchyma by the int rvening pia mat r or arachnoid and pia
mater. Grafting to the ventricle may be accomplished by

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injection of the donor c lls or by growing th cells in a
substrate such as 3% collagen to form a plug of solid tissue
which may then b implant d into the v ntricle t prevent
dislocation of the graft. For subdural grafting, the cells may

5   be injected around the surface of the brain after making a
slit in the dura. Injections into selected regions of the
host brain may be made by drilling a hole and piercing the
dura to permit the needle of a microsyringe to be inserted.
The microsyringe is preferably mounted in a stereotaxic frame

10   and three dimensional stereotaxic coordinates are selected for
placing the needle into the desired location of the brain or
spinal cord.

        The donor cells may also be introduced into the

15   putamen, nucleus basalis, hippocampus cortex, striatum or
caudate regions of the brain, as well as the spinal cord.

        Preferably, for passaged donor cells, cells are passaged
from approximately 2 to approximately 20 passages.

20

        For grafting, the cell suspension is drawn up into the
syringe and administered to anesthetized graft recipients.
Multiple injections may be made using this procedure. The age
of the donor tissue, i.e. the developmental stage may affect,

25   the success of cell survival after grafting.

        The cellular suspension procedure thus permits grafting
of genetically modified donor cells to any predetermined site
in the brain or spinal cord, is relatively non-traumatic,

30   allows multiple grafting simultaneously in several different
sites or the same site using the same cell suspension, and
permits mixtures of cells from different anatomical regions.
Multiple grafts may consist of a mixture of cell types, and/or
a mixture of transgenes inserted into the cells.

35   Preferably from approximately 10^6 to approximately 10^8 cells
are introduced p r graft.

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For transplantation int cavities, which may b pr f rred for spinal cord grafting, tissue is removed from r gi ns close to the xternal surfac of the CNS to form a transplantation cavity, for example as described by Stenevi et al., supra, by

5   removing bone overlying the brain and stopping bleeding with a material such a gelfoam. Suction may be used to create the cavity. The graft is then placed in the cavity. More than one transplant may be placed in the same cavity using injection of cells or solid tissue implants.

10

Grafting of donor cells into a traumatized brain will require different procedures, for example, the site of injury must be cleaned and bleeding stopped before attempting to graft. In addition, the donor cells should possess sufficient

15   growth potential to fill any lesion or cavity in the host brain to prevent isolation of the graft in the pathological environment of the traumatized brain.

The present invention therefore provides methods for

20   genetically modifying donor cells for grafting CNS to treat defective, diseased and/or injured cells of the CNS.

The methods of the invention also contemplate the use of grafting of transgenic donor cells in combination with other

25   therapeutic procedures to treat disease or trauma in the CNS. Thus, genetically modified donor cells of the invention may be co-grafted with other cells, both genetically modified and non-genetically modified cells which exert beneficial effects on cells in the CNS, such as chromaffin cells from the adrenal

30   gland, fetal brain tissue cells and placental cells. The genetically modified donor cells may thus serve to support the survival and function of the co-grafted, non-genetically modified cells, for example fibroblasts modified to produce nerve growth factor (NGF) in vivo as described in the

35   Examples, infra.

Mor ov r, the g netically modified donor c lls f the

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inventi n may be co-administ r d with therap utic ag nts
useful in treating d fects, trauma or diseas s of the CNS,
such as growth factors, e.g. n rve gr wth fact r;
gangliosides; antibiotics, neurotransmitters, neurohormones,

5   toxins, neurite promoting molecules; and antimetabolites and
precursors of these molecules such as the precursor of
dopamine, L-dopa.

       The methods of the invention are exemplified by preferred
10   embodiments in which donor cells containing vectors carrying a
therapeutic transgene are grafted intracerebrally into a
subject. In a first preferred embodiment, the established
HPRT-deficient rat fibroblast line 208F, primary rat
fibroblasts, and day-1 postnatal primary rat astrocytes were

15   used to demonstrate that cultured cells genetically modified
using retroviral vectors can survive when implanted in the
mammalian brain and can continue to express transgene
products.

20   In a second preferred embodiment fibroblasts were
genetically modified to secrete NGF by infection with a
retroviral vector, and the modified fibroblasts were then
implanted into the brains of rats with surgical lesions of the
fimbria fornix region. The grafted cells survived and

25   produced sufficient NGF to prevent the degeneration of
cholinergic neurons that would die without treatment. In
addition, the protected cholinergic cells sprouted axons that
projected in the direction of the cellular source of NGF.

30   In a third preferred embodiment fibroblasts were
genetically modified to express and secrete L-dopa by
infection with a retroviral vector, and the modified
fibroblasts were grafted into the caudate of rats modeling
Parkinson's disease as a result of unilateral dopamine

35   depletion. The cells survived and produced sufficient L-dopa
to decreas the rotati nal movement caus d by dopamin
d pleti n.

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In a fourth preferred embodiment primary skin fibroblasts
isolated from skin biopsi s and maintained in culture were
employ d as autologous cells for intracerebral grafting into
the adult rat striatum. The fibroblasts ceased to proliferate
5   once they reached confluency and were contact inhibited _in
vitro. The fibroblasts were able to survive for at least
eight weeks following intracerebral implantation and continued
to synthesize collagen and fibronectin _in vivo_. The grafts
also maintained a constant volume between three and eight
10   weeks, indicating that the primary skin fibroblasts did not
tumor or die. Dynamic host-to-graft interactions, including
phagocytic migration, astrocytic hypertrophy and infiltration
into the grafts, and angiogenesis, were observed indicating
the structural integration of grafts of primary skin
15   fibroblasts in the adult rat CNS.

In a fifth preferred embodiment primary skin fibroblasts
obtained from a skin biopsy from an inbred strain of rats were
used as donor cells for genetic modification and grafting.
20   When grafted into the brain of rats of the same genetic strain
as the donor rats, the fibroblasts containing a transgene for
either tyrosine hydroxylase (TH) or β-galactosidase survived
for 10 weeks and continued to express the transgene. The TH
synthesized by the implanted fibroblasts appeared to convert
25   tyrosine to L-dopa, as observed _in vitro_, and to affect the
host brain as assessed through a behavioral measurement.
Supplying L-dopa locally to the striatum was shown to be
sufficient to partially compensate for the loss of striatal
dopaminergic input.
30

In a sixth preferred embodiment, the ability of aged
human fibroblasts to serve as donor cells for human NGF was
demonstrated.

35   In a seventh preferred embodiment rat fibroblasts were
genetically modifi d t expr ss and secrete choline
ac tyltransferase (dChAT). It was shown that intra- and

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extracellular levels f ACh c uld be increased by adding
exogenous choline chloride. In addition, serum starvation or
confluenc -induced quiescence caused an 80% d crease in
recombinant ChAT activity (as compared to actively growing

5   cells). ACh release was also repressed in quiescent
fibroblast cultures. Exogenous choline mitigated the decrease
in ACh secretion. These results indicated that fibroblasts
can be genetically modified to produce ACh and that ACh
release can be regulated, for example increased, by

10 introducing choline into the culture medium.

In an eighth preferred embodiment selected promoters
including collagen promoters were demonstrated to increase
expression of the transgene chloramphenicol acetyltransferase

15 (CAT) in quiescent primary fibroblasts.

In a ninth preferred embodiment cytokines and an anti-
inflammatory agent, dexamethasone, were evaluated for their
effect on LTR promoter activity to enhance expression of the

20 CAT and dChAT genes in primary skin fibroblasts. In addition,
the collagen promoter α2(I) with the collagen enhancer
(Coll(E)) was used to evaluate the effects of cytokines on the
expression of CAT in quiescent fibroblasts.

25 In an tenth preferred embodiment primary skin fibroblasts
were modified to express and secrete NGF and were grafted into
the striatum of adult female rats. At one and three weeks
following striatal implantations, NGF receptor-immunoreactive
axons surrounded the grafts of NGF-producing fibroblasts. At

30 three and eight weeks, NGF receptor-positive profiles were
also found within the grafts. Control grafts of normal
primary fibroblasts lacked immunoreactive axons.
Ultrastructural examination showed that unmyelinated axons
within the extracellular matrix of the grafts were enveloped

35 within processes of reactive astrocytes and a distinct basal
lamina surround d the axo-glial bundles. Processes of
reactiv astrocyt s were evident in b th c ntrol and grafts

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containing genetically modified primary fibr blasts. This
embodiment dem nstrates an _in vivo_ model showing that the
release of NGF from the grafts induced directi nal growth of
NGF receptor-positive axons and that mature reactive

5   astrocytes provide a permissive substrate upon which axons
migrated. In this embodiment, genetically modified
fibroblasts were also placed in a collagen matrix graft to
assess the regenerative capacity of the adult rat medial
septum. The grafts produced NGF and promoted the regeneration

10   of septal axons; the grafts possessed large numbers of
unmyelinated axons compared to control fibroblast grafts. The
regenerating septal axons provided a reinnervation to the
deafferented hippocampus; the topographical and synaptic
organization of the septal inputs within the hippocampal

15   dentate gyrus was similar to that of the normal cholinergic
innervation arising from septal nuclei.

20   In order that the invention described herein may be more
fully understood, the following examples are set forth. It
should be understood that these examples are for illustrative
purposes only and are not to be construed as limiting the
scope of this invention in any manner.

25

Example I

Intracerebral Grafting Of Genetically
Modified Cells Expressing HPRT
30   Transgene To The Brain

Infection of Cells

35   Donor hypoxanthine guanine phosphoribosyl transferase
(HPRT)-deficient 208F rat fibroblast cells (Jolly et al.,
Proc. Natl. Acad. Sci. USA 80:477-481 (1983)) were infected
with the pr t type HPRT vect r pLS P LM expressing HPRT cDNA
(Miller et al., _Science_ 225:630-632 (1984); Yee et al., _Gene_
40   53:97-104 (1987)) cDNA r with th neo^r-luciferase v ct r

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pLNHL2 (Eglitis et al., Science 230:1395-1398 (1985); W lff et
al., Proc. Natl. Acad. Sci. (USA) expressing b th the Tn5
transpos n neomycin-resistance gene (ne R,) and the firefly
luciferase cDNA (de Wet et al., Molec. Cell. Biol. 7:725-737

5   (1987)) (Figure 4). The pLS P LM vector was derived from
vector pLPL2 (Miller et al., Molec. Cell. Biol. 6:2895-2902
(1986)) and contains human HPRT cDNA encoding a protein with a
novel C-terminal hexapeptide added by in vitro mutagenesis of
the translational termination codon (Yee et al., Gene

10   53:97-104 (1987)). Vector pLS P LM was constructed as
follows: Vector pLpLM (Yee et al., Gene 53:97-104 (1987)) was
digested with XhoI and BamHl to yield a 1.3 Kb fragment which
was then ligated into plasmid pLpL2 (Miller, supra) which had
also been restricted using XhoI and BamHl. The resulting

15   vector was pLS P LM.

The vector pLNHL2 contained the cDNA encoding the firefly
luciferase (LUX) and the Tn5 neomycin-resistance gene (neo^R)

20   and the promoter and enhancer of the human cytomegalovirus
immediate early gene (HCMV). The 5' and 3' LTRs were derived
from cloned murine leukemia virus (MLV) as described by Mason
et al., in Science 234:1372-1378 (1986). Vector pLNHL2 was
constructed as follows: Plasmid pLNHPL2 (also known as

25   PNHP-1, Yee et al., Proc. Natl. Acad. Sci. USA 84:5197-5209
(1987)) was restricted with BamHI to remove the HPRT DNA
sequence. The ends were repaired using Klenow polymerase.
Plasmid pSV2A (deWet et al., Molec. Cell. Biol., supra, and
supplied by) (Dr. Subramani, University of California, San

30   Diego, CA) was restricted with HindIII and Sspl to isolate the
luciferase fragment. The ends were repaired as above. The
BamHl restricted pLNHL2 and HindIII-Sspl restricted pSV2A were
ligated together forming vector pLNHL2 (Fig. 5).

35   The cells were grown in selective medium containing
hyp xanthine, aminopterin and thymidin (HAT) f r c lls
expr ssing HPRT and with the n omycin analog G418 f r cells

expressing n ^R, r sp ctively, to nsur that only inf cted
cells were us d.

5   Primary fibroblasts and astrocytes were infected with the
neo^R-luciferase vector only. HAT-resistant and G418-resistant
cells were harvested following incubation overnight with
serum-free medium or medium containing rat serum, to reduce
the likelihood of immunological response in the rat brain.

10   Grafting

The cells were resuspended in a balanced glucose-saline
solution and injected stereotaxically into several regions of
the rat brain using a sterile syringe. Between 10,000 and
15   100,000 cells per microliter were injected at a rate of
1 μl/min for a total volume of 3-5 μl. After 1 week to 3
months the animals were killed and areas containing the
implanted cells were identified, excised, and examined
histologically and biochemically.
20

Histological Analyses

To evaluate the grafted cells histologically, the rats
were perfused transcardially and their brains were sectioned
25   and stained with Nissl stain and cresyl violet for general
morphological characterization and with immunocytochemical
methods to establish the presence of the specific cell
antigenic markers fibronectin for the fibroblasts and glial
fibrillary acidic protein (GFAP) for glial cells. Briefly,
30   the sections were rinsed in Tris-buffered saline (TBS)
solution (pH 7.4) containing 0.25% Triton-X. The sections
were incubated for 24 hrs at 4°C with rabbit polyclonal
antibodies to fibronectin (1:2000 dilution; Baralle,
University of Oxford, England) and GFAP (Gage et al., Exp.
35   Neurol. 102:2-13 (1989); available from Dakopatts, Glostryp,
Denmark) dilut d 1:1000 in TBS containing 0.25% Trit n-X and
3% g at serum r with the m nocl nal antibody, mous IgG2a,

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against a membrane polypeptide of rat macr phages,
granul cyt s and d ndritic cells (MRC OX-42, Ser tec) dilut d
1:100 in TBS c ntaining 0.25% Triton-X and 1% h rs s rum.
After thorough rinsing, the sections were incubated for 1 hr

5   with biotinylated goat anti-rabbit IgG (Vectastain) diluted
1:200 in 0.1 M TBS containing 0.25% Triton-X and 15 horse
serum, followed by several rinses in TBS containing 0.25%
Triton-X and 1% goat serum or 1% horse serum. The sections
were then incubated for 1 hr at room temperature with a

10   complex of avidin and biotinylated horseradish peroxidase
(Vectastain, ABC kit, Vector Labs, Burlingame, CA) diluted
1:100 in 0.1 M TBS containing 0.25% Triton-X and 1% goat serum
or 1% horse serum, followed by thorough rinses. The
peroxidase was visualized by reacting with 0.05%,

15   3,3-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical
Co., St. Louis, MO) and 0.05% $NiCl_2$ and 0.01% H_2O_2 in TBS for
15 min at room temperature.

Primary rat fibroblasts grafted to the neostriatum of the
20   rat seven weeks earlier are illustrated in Fig. 6. Serial 40
μm-thick sections were stained with antifibronectin (Fig. 6a,
Fig. 6d), cresyl violet (Disbrey et al., Histological
Laboratory Methods, E. & S. Livingstone, Edinburgh and London
(1970)), (Fig. 6b, Fig. 6e) and anti-GFAP (Fig. 6c, Fig. 6f).
25   The surviving cells appeared to be intact and to have clumped
or aggregated around the area of the injection. The cells
displayed an intense staining for fibronectin at the core of
the graft, with a clear GFAP-staining derived from reactive
gliosis at the edges of the grafts, similar to what one sees
30   with the cannula tract alone. However, little GFAP-staining
was observed in the graft itself. With cresyl violet, small,
round, darkly stained cells were observed in the region of the
graft which could either be microglia or lymphocytes that had
infiltrated the area in response to injury. Macrophages could
35   also be detected in many of the grafts. Many of the
fibr blasts could be id ntifi d by cr syl vi let staining by
their l ng thin shape and by th pink pleated sheets f

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collagenous mat rial surr unding them. The appearance f 208F
fibroblasts was similar t the primary fibroblasts (n t
shown). Astrocyte grafts also had a similar appearance,
except they were not fibronectin-positive, and stained for
5   GFAP through the center of the grafts. For all three cell
types, no differences were observed between
retrovirus-infected cells and control cells. An important
feature of these cell suspension grafts is that most of the
cells remained aggregated near the site of injection and did
10   not appear, under these circumstances, to migrate very far
from the injection site into the host brain. This apparent
lack of migration could certainly be different for other donor
cell types and graft sites, and therefore the area of the
brain into which the cells are to be implanted, the nature of
15   the donor cells, and the phenotype of. the target cells for the
transgene may be important factors for the selection of donor
cells.

Characterization of Implanted Cells
20

      Implanted cells were dissected out and prepared for
reculturing and for biochemical and molecular character-
ization by dissociating the cells with trypsin. For the
detection of the human HPRT activity, cell extracts were
25   prepared from the bulk of each sample as previously described
and examined by a polyacrylamide gel isoelectric focusing HPRT
assay (Jolly et al., _Proc. Natl. Acad. Sci. USA_) 80:477-481
(1983); Miller et al., _Proc. Natl. Acad. Sci (USA)_
80:4709-4713 (1983); Willis et al., _J. Biol. Chem._
30   259:7842-7849 (1984); Miller et al., _Science_ 225:630-632
(1984); Gruber et al., _Science_ 230:1057-1061 (1985), and Yee
et al., _Gene_ 53:97-104 (1987)). The remainder of each sample
was placed into culture. The results of an HPRT gel assay of
rat 208F cells HAT resistance after infection with the HPRT
35   vector implanted into one side of the rat basal ganglia 3 and
7 we ks after transplantation and pri r t analysis are
shown in Fig. 7.

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The presence of human HPRT enzyme activity dem nstrates
that th infected, g netically modified rat 208F cells grafted
int the brain survived and continued t express th HPRT
transgene at easily detectable levels for at least 7 weeks.

5   Furthermore, the implanted cells could be successfully
recultured, producing cells morphologically identical to the
starting cultures. Infection of these cells with helper virus
resulted in the production of HPRT virus, confirming the
identity of the cells and indicating that the provirus

10   remained intact. Studies with the neo^R-luciferase vector
confirm the survival and expression of luciferase-infected
cells.

<u>Example II</u>

15   Grafting of Genetically Modified Cells
Expressing NGF to the Damaged Brain

The above example demonstrated that cultured cells

20   genetically modified using retroviral vectors can survive when
implanted into the mammalian brain and can continue to express
transgene product. The present example was conducted to
determine whether sufficient transgene product can be made by
genetically modified cells <u>in vivo</u> to complement or repair an

25   absent or previously damaged brain function.

<u>Construction of NGF Vector pLN.8RNL</u>

A retroviral vector, similar to one described previously

30   (Wolf, et al., <u>Mol. Biol. Med</u>. 5:43-59 (1988)), was
constructed from Moloney murine leukemia virus (MoMuLV)
(Varmus et al., <u>RNA Tumor Viruses</u>; Weiss et al., Cold Spring
Harbor Press, Cold Spring Harbor, NY, p. 233 (1982)). The
pLN.8RNL vector contains the 777 base pair Hgal-Pstl fragment

35   of mouse NGF cDNA (Scott et al., <u>Nature</u> 302; 538 (1983);
Ullrich et al., <u>Nature</u> 303:821 (1983)), under control of the
viral 5' LTR. This insert corresponds to the shorter NGF
transcript that pr d minat s in m use tissu rec iving
sympathetic innervati n (Edwards t al., <u>Nature</u> 319:784

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(1986)) and is believ d to ncod th pr cursor t NGF that is
secr ted constitutively. The v ctor als included a dominant
s lectable marker encoding th neomycin-r sistanc functi n f
transposon Tn5 (Southern et al., *J. Mol. Appl. Genet*. 1:327
5   (1982)), under control of an internal Rous sarcoma virus
promoter.

Plasmid pSPN15' (Wolf et al., *Mol. Biol. Med.* 5:43-59
(1988), supplied by Dr. Breakefield, Harvard Medical School,
10   Boston, MA) was digested with restriction enzymes Pstl and
Hgal using established methods (Maniatis et al., Cold Spring
Harbor Press, Cold Spring Harbor, New York, (1982)) and the
777 base pair (bp) DNA fragment containing the NGF sequences
was isolated by standard purification methods (Maniatis, et
15   al., *supra*). The 777 bp fragment was then blunt-end ligated
into plasmid pLMTPL as described by Yee et al., in *Proc. Natl.
Acad. Sci. (USA)* 84:5197-5201 (1987), incorporated by
reference herein. Plasmid pLMTPL was digested with Hind III
to remove the metallothionein promoter and most of the HPRT
20   cDNA, and the overhanging 5' ends were repaired using Klenow
polymerase as described by Maniatis et al., *supra*. The
overhanging ends of the 777 bp fragment isolated as above were
similarly repaired. The 777 bp fragment was then blunt end
ligated into the digested plasmid pLMTPL. The resulting
25   plasmid was called PLN.8L and was transfected into *E. coli*
strain DH1, grown and purified by established methods for
plasmid purification including cesium chloride centrifugation
(Maniatis et al., *supra*) . The purified plasmid pLN.8L was
then digested using restriction enzymes BamHI and Clal and the
30   resulting 6.1 kilobase fragment was ligated to a 2.1 kilobase
BamHl-Clal fragment isolated from plasmid pLLRNL. Plasmid
pLLRNL was derived from plasmid JD204 described by de Wet et
al., in *Mol. Cell. Biol*. 7:725-737 (1987) as follows: A 1717
bp HindIII-Sspl fragment from the firefly luciferase gene
35   derived from plasmid JD204 and a 1321 bp HindIII-Smal fragment
of the plasmid pSV2Neo[R] describ d by S uthern and B rg in *Mol.
Appl. Genet*. 1:327-341 (1982) wer ligated with a fragment

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containing a mutat d RSV prom ter in a 300 bp BamHI-HindIII
fragment from plasmid pUCRH. Plasmid pLLRNL is depicted in
Fig. 8.

5

        Plasmid pUCRH was produced as follows. Plasmid pRSVneo[R]
was restricted using HindIII and the linearized plasmid was
ligated with the remaining fragment from plasmid pPR1 (Fig. 9)
(supplied by Dr. Friedmann, University of California, San

10 Diego, CA) obtained by restriction using HindIII to remove
HPRT sequences. Plasmid pPR1 was obtained from plasmid p4aA8
(Jolly et al., Proc. Natl. Acad. Sci. 80:477-481 (1983)) using
Pst and Rsa. The resulting plasmid was called pRHN and was
then restricted with PvuII and religated to form plasmids

15 PN(+) and PAC(-). PN(+) was then restricted using BamH1. The
resulting linearized plasmid was ligated with a fragment
obtained from plasmid pSVori restricted with BamHI. Plasmid
pSVori was obtained by restricting plasmid p4aA8 with Sall nd
Pst1, and subcloning the resulting fragment into plasmid pUC18

20 (Bethesda Research Laboratories, Gaithersburg, MD) that had
been restricted with Sall and Pstl. The resulting plasmid was
termed pSVori.

        The plasmid pRH+S+ that resulted from ligation of the

25 BamH1 restricted plasmid PN(+) and the BamH1 restricted
plasmid pSVori was then restricted with MstII and the
overhanging 5' ends were repaired using Klenow polymerase as
described above. This fragment was ligated with a M13mp18
(Bethesda Research Laboratories) linearized with Smal and

30 phosphatased with calf intestinal alkaline phosphatase
(Boehringer Mannheim, Mannheim, W. Germany). The resulting
plasmid was called pmpRH and contained the HPRT cDNA expressed
from the RSV promoter.

35   Plasmid pmpRH was subject to site-directed mutagenesis as
described by Kunkel et al., Proc. Natl. Acad. Sci. USA
82:488-492 (1985), incorporat d by reference herein in rd r

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t alter the polyadenylati n signal AATAAA to AGCAAA. After
mutagenesis the resulting plasmid was restricted using HindIII
and th r sulting fragm nt was ligated to a HindIII fragment
from restriction of plasmid pUC19 (Bethesda Research

5   Laboratories) to produce plasmid pUCRSV. Plasmid pUCRSV was
restricted using BamH1 and Pst1 to produce a fragment
containing the RSV promoter. This fragment was ligated to a
Pst1-Sst1 fragment containing the gene encoding HPRT obtained
by restriction of the plasmid pLS P LM as described in Example

10   I, supra and to a BamHI-Sst1 fragment obtained from plasmid
pUC19, forming plasmid pUCRH (Fig. 10). The product of
ligation between the BamH1-Hindlll fragment from pUCRH, the
1717 bp HindIII-Sspl fragment from pJD204 and the 1321 bp
HindIII-Smal fragment from pSV2Neo^R was transfected and

15   purified by established methods as described above and termed
plasmid pLLRNL (Fig. 8).

        . After transfection and purification, the plasmid
resulting from ligation of the 6.1 BamH1-Clal kilobase

20   fragment from plasmid pLN.8L and the 2.1 kilobase BamH1-Clal
fragment from plasmid pLLRNL was termed pLN.8RNL.   (Figs. 11
and 12).

Preparation of Transmissible Retrovirus

25

        Transmissible retrovirus was produced by transfecting
pLN.8RNL into PA317 amphotropic producer cells (Miller et al.,
Mol. Cell. Biol. 6:2895 (1986)), supplied by Dr. Miller, Fred
Hutchinson Cancer Research Center, Seattle, WA), by the

30   calcium phosphate co-precipitation method (Graham et al.,
Virology 52:456 (1973)), and using medium from these cells to
infect Ψ-2 ecotropic producer cells (Mann et al., Cell 33:153
(1983)) in the presence of 4 μg/ml Polybrene (Sigma Chemical,
St. Louis, Mo). Virus from the Ψ2 clone producing the highest

35   titer, 4 x 10^5 colony forming units/ml, was used to infect the
established rat fibr blast c 11 line 208F (Quade, Virology
98:461 (1979) as described by Miyanohara et al., in Proc.

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Natl. Acad. Sci. USA (1988)).

Assay for NGF Production and Secretion

5   Individual neomycin-resistant 208F colonies, selected in
medium containing the neomycin analog G418, were expanded and
tested for NGF production and secretion by a two site (ELISA)
enzyme immunoassay (Korsching et al., Proc. Natl. Acad. Sci.
(USA) 80:3513-3516 (1983)), using commercially available
10   reagents according to the manufacturer's protocol (Boehringer
Mannheim). The clone producing the highest levels of NGF
contained 1.7 ng NGF/mg total cellular protein and secreted
NGF into the medium at a rate of 50 pg/hr 10^5 cells. The NGF
secreted by this clone was biologically active, as determined
15   by its ability to induce neurite outgrowth from PC12 rat
pheochromocytoma cells (Greene, et al., Proc. Natl. Acad. Sci.
USA 73:2424 (1976)). Uninfected 208F cells, in contrast, did
not produce detectable levels of NGF in either assay.

20   ## Fimbria Fornix Transection

          Fimbria fornix transection (axotomy) was performed as
described by Gage et al., Brain Res. 268:27-37 (1983) and in
Neuroscience 19(1) 241-255 (1986), both of which are
25   incorporated by reference herein. Briefly, adult female
Sprague-Dawley rats (Bantin and Kingman, San Francisco, CA)
weighing between 200g and 225g at the beginning of the
experiment were used. The animals were anesthetized with
intraperitoneal injections of a ketamine-xylazine mixture (10
30   μg/kg Ketalar, Parke-Davis Ann Arbor, MI, and 5 μg/kg Rompun,
Hoechst, Frankfurt, W. Germany). Unilateral aspirative
lesions were made by suction through the cingulate cortex,
completely transecting the fimbria fornix unilaterally, and
removing the dorsal tip of the hippocampus as well as the
35   overlying cingulate cortex to inflict a partial denervation on
th hippocampus target, as d scribed in Gage et al., Brain
Res. 268:27-37 (1983). All animals in each of the

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experimental groups received the same complete unilateral aspirative lesi n. Fimbria fornix lesi ns as described ab ve were made in 16 rats; 8 rats rec ived grafts of infected c lls while the remaining 8 received uninfected control cells.

5

Sections stained for fibronectin, a fibroblast specific marker, revealed robust graft survival that was comparable in both groups (Fig. 13a, Fig. 13b). Sections stained for choline acetyltransferase (ChAT) to evaluate the survival of

10 cholinergic cells bodies indicated a greater number of remaining neurons on the lesioned side of the medial septum in animals that had received grafts of infected cells than in animals that had received control grafts (Fig. 13c-Fig. 13f).

15 Retrovirus-infected (NGF secreting) and control 208F cells were removed from confluent plates with Dulbecco's phosphate buffered saline (PBS) containing 0.05% trypsin and 1 mM EDTA and taken up by trituration with PBS supplemented with 1 mg/ml glucose, 0.1 mg/ml each $MgCl_2$ and $CaCl_2$ (complete PBS)

20 and 5% rat serum to inactivate the trypsin. Cells were pelleted by centrifugation at 1000 X g for 4 min. at 4°C, washed twice with complete PBS, and resuspended in complete PBS at 10^5 cells/μl. Four μl of suspended cells were injected free-hand using a Hamilton syringe into the cavity and lateral

25 ventricle ipsilateral to the cavity in the animals. A piece of Gelfoam was gently placed on the surface of the cavity and the animals were sutured.

<u>Immunohistochemistry</u>

30

At 2 weeks following surgery the rats were perfused and their brains were removed, fixed overnight and placed in phosphate-buffered 30% sucrose for 24 hr at 4°C. Sections 40 μm thick were cut on a freezing sliding microtome and

35 stored in cryoprotectant (phosphate-buffered glycerol and ethylene glycol) at -20°C. Every fifth secti n was labelled immunohistochemically by standard procedures using p lycl nal

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antibodies t fibronectin t evaluate fibr blast survival.
Polyclonal antibodies to choline acetyltransferase (anti-ChAT
antiserum) wer als generated t evaluate the survival f
cholinergic cell bodies as described by Gage et al. in _J. of
5   _Comparative Neurol_. 269:147-155 (1988), incorporated by
reference herein. Tissue sections were processed for
immunohistology according to a modification of the
avidin-biotin labeling procedure of Hsu et al.,
29:1349-1353(1981), incorporated by reference. This procedure
10   consists of the following steps: 1) overnight incubation with
antibody to ChAT or with control antibody (i.e. preimmune
serum or absorbed antiserum). The ChAT antibody was diluted
1:1,500 with 0.1 M Tris-saline containing 1% goat serum and
0.25% Triton X-100; 2) incubation for 1 hr with biotinylated
15   goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA)
diluted 1:200 with Tris-saline containing 1% goat serum: 3)
1 hr incubation with ABC complex (Vector Laboratories) diluted
1:100 with Tris-saline containing 1% goat serum; 4) treatment
for 15 min with 0.05% 3,3'diaminobenzidine (DAB), 0.01%
20   hydrogen peroxide and 0.04% nickel chloride in 0.1 M Tris
buffer. Immunolabeled tissue sections were mounted onto glass
slides, air dried and covered with Permount and glass
coverslips. Two sections stained for ChAT through the septum,
200 μm apart were used to evaluate the extent of cholinergic
25   cell survival. All the ChAT-positive cells in the ipsilateral
septum and in the contralateral septum were counted separately
and sized for planar area using an Olympus Que-2 image
analysis system. Tissues were also stained for
acetylcholinesterase (AChE) as described by Hedreen et al.,
30   _J. Histochem. Cytochem_. 33:134-140 (1985), incorporated by
reference herein, to evaluate the completeness of the fimbria
fornix transection.

Neuronal survival was quantitated (Fig. 14) and, when
35   expressed as a percentage of the remaining cholinergic cells
in the septum ipsilateral to the lesion r lativ to the intact
contralateral septum, was shown t be 92% in animals grafted

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with NGF-secreting cells but only 49% in animals grafted with
contr l cells. The results from the control gr up are
comparable to previous observations in lesioned animals that
had received no grafts (Gage et al., Neuroscience 19:241

5   (1986); Hefti, J. Neurosci. 8:2155 (1986); Williams et al.,
Proc. Natl. Acad. Sci. USA 83:9231 (1986); Kromer, Science
235:214 (1987); Gage et al., J. Comp. Neurol. 369:147 (1988)).

In addition to the significant increase in the percentage
10   of ChAT-positive cells in the NGF group, these animals also
showed an increase in AChE-positive fiber and cell staining
(Fig. 15). Most striking was the observation of a robust
sprouting response in the dorsal lateral quadrant of the
septum, with the most intense staining abutting the cavity
15   containing the graft. This intense increase in AChE staining
was not observed in the group receiving control grafts (Fig.
15).

The above results demonstrate the feasibility of
20   continued transgene expression by cells grafted to the CNS and
also present the first demonstration of a phenotypic
correction in whole animals brought about by grafted,
genetically modified cells.

Example III

25

Grafting of Genetically Modified Fibroblasts
Expressing L-dopa Into The CNS of A Rat
Model of Parkinson's Disease

30   This example was undertaken to demonstrate that the
methods of the present invention for genetic modification of
donor cells and grafting of the cells into the CNS can

significantly ameliorate the signs of disease in an animal
35   model, such as a rat model of Parkinson's disease.

The strategy for enabling fibroblasts to produce L-d pa
used in this example is based upon the ability of the enzyme

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tyrosin hydroxylase (TH) t catalyze the conversi n of
tyrosine t L-dopa; th rat -limiting step in cat ch lamine
synthesis. Tetrahydrobiopterine (H_4-B), the c -factor for TH
is required for TH enzymic activity. Since the brain contains

5   significant levels of biopterin, and fibroblasts can reduce
biopterin to H_2-biopterin, TH should be active in fibroblasts
situated within the brain.

Construction of Retroviral Vector pLThRNL

10

The vector pLThRNL, a Moloney leukemia virus (MoMLV)
derived retroviral vector, was constructed expressing the rat
cDNA for tyrosine hydroxylase (TH) from the 5' LTR sequence
and contained a neomycin-resistant gene transcribed from an

15   internal RSV promoter (Fig. 16). Fragments from three
plasmids: pLRbL, pTH54 and pLHRNL were ligated together to
form pLThRNL. Plasmid pLRbL was obtained by digesting plasmid
pLMTPL (obtained as described above in Example II) with the
enzymes HindIII and Hpal, and removing the fragment containing

20   the HPRT gene. The remaining plasmid DNA was ligated with the
3.5 kb fragment obtained after restriction of plasmid
pGEM1-4.5Rb old (pGEM1-4.5Rb old was constructed by inserting
DNA encoding the retinoblastoma gene (Rb) into plasmid pGEM1,
available from Promega, Madison, WI, and was supplied by Dr.

25   Lee, University of California, San Diego, CA) using HindIII
and Sca2. The resulting plasmid was named pLRbL.

A 1688 bp fragment containing rat TH cDNA was obtained
from the plasmid pTH54 (O'Malley, *J. Neurosci. Res*. 60:3-10

30

(1986), supplied by Dr. O'Malley, Washington University, St.
Louis, MO) by digestion with BamIII and Sphl.

The fragments from plasmid pTH54 and plasmid pLRbL were

35   ligated with a Cla1 and Smal fragment obtained from plasmid
pL2RNL (described above in Exampl I) to obtain the v ct r
pLThRNL c ntaining the retroviral provirus f r transfection

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int producer cells to produce virus carrying the g ne
encoding the enzyme tyr sine hydroxylase. The derivation of
and circular restriction map for pLThRNL is shown in Fig. 17.

5   Helper-free retrovirus was produced and retroviral
infections were done as described in Example II, supra
Plasmid DNA containing the LThRNL provirus was CaPO$_4$
transfected as described by Wigler et al., Cell 11:223-232
(1977), incorporated by reference herein, into the amphotropic
10   PA317 helper line supplied by Dr. Miller, Fred Hutchinson
Cancer Research Center, Seattle, WA. Two days
post-transfection, media from these cells were filtered and
used to infect the ecotropic Ψ2 helper line (Miller et al.,
Mol. Cell. Biol. 6:2895-2902 (1986), supplied by Dr. Miller).
15   A G418-resistant Ψ2 clone (Ψ2/TH) that contained the highest
level of TN activity and produced the highest titre of virus
(5 x 10^5/ml) was selected. Immortalized, rat fibroblasts
(208F) (Quade, Virology 98:461-465 (1979)) were infected at a
multiplicity of infection (MOI) of less than 10^{-4} with LThRNL
20   virus produced by the Ψ2/TH producer cells. G418 resistant
clones were established for further study. All retroviral
infections were done in the presence of 4 µg of Polybrene
(Sigma) per ml. Cells were selected for expression of the
neomycin-resistance gene by growth in 400 µg/ml of G-418.

25

Assay of Tyrosine Hydroxylase Activity

30

        Confluent 10 cm plates of cells were washed two times
with Dulbecco's phosphate buffered saline (PBS) not containing
calcium or magnesium chloride and the cells were scraped off
the plates. The cells were homogenized in 0.15 ml of ice cold
35   50 mM Tris/50 mM sodium pyrophosphate/0.2% Triton X-100,
adjusted t pH 8.4 with ac tic acid, and wer c ntrifuged at
32,000 X g for 15 min at 2-4° C. The supernatant fraction was

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used f r both TH and protein measur ments. TH activity was
measured with a decarboxylase-coupled assay essentially as
d scribed previously (Iuvone et al., J. Neurochem, 43:
1359-1368 (1986)), but with ^{14}C-labelled 20 μM tyrosine, 1 mM

5   6-methyl-5,6,7,8-tetrahydropterin (6MPH4) (Calbiochem, La
Jolla, CA), and potassium phosphate buffer (pH 6). Protein was
determined by the method of Lowry et al. (J. Biol. Chem.
193:265 (1951)) using bovine serum albumin as standard.

10   Assays of Catecholamines and their Metabolites

Cultured cells were scraped off plates as in the assay of
tyrosine hydroxylase activity except ascorbic acid (final
concentration of 50 nM) was added to the cell pellets prior to

15   freezing. Cells were grown in DME plus 10% fetal calf serum.
Some cultures were supplemented with 0.1 mM 6MPH$_4$, 16 hours
prior to harvesting. Cells were homogenized in 250 μl of ice
cold 0.1 N HCl/0.1% sodium metabisulfite/0.2% Na$_2$ EDTA
containing 5 ng/ml of 3,4-dihydroxybenzylamine (DHBA) as

20   internal standard. Dopamine, DOPA, 3,4-dihydroxyphenylacetic
acid (DOPAC), DHBA and 3-methoxy-4-hydroxyphenylglycol (MHPG),
were extracted from the supernatant fraction by alumina
absorption (Anton et al., J. Pharmacol. Exp. Ther. 138:360-375
(1962)) and eluted with 150 μl of 0.1N H$_3$PO$_4$. They were

25   analyzed by HPLC with electrochemical detection as described
by Iovone et al., Brain Res. 418:314-324 (1987), with the
mobile phase modified to contain a higher concentration of
sodium octylsulfate (0.45 mM) and lower pH (2.8).
Homovanillic acid (HVA) was analyzed by HPLC. The

30   concentration of catecholamines and metabolites in the media
was also determined using a different method, HPLC-EC. The
alumina extraction procedure described above was omitted and
the media was adjusted to 0.1 perchloric acid/0.01 M EDTA was
centrifuged 10,000g x 10 min to remove precipitated material

35   and used directly for HPLC-EC. In this system, the whole
phase consisted f 0.137% SDS in 0.1 M phosphate buff r, pH
3.2 (Buffer A) or 40% methan l in 0.1 M phosphate buffer, pH

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3.35 (Buffer B). C mpounds in sample wer eluted for 12
minutes in 100% Buffer A, foll wed by a gradi nt increasing
linearly ov r 30 minutes to 100% Buffer B. The eluant was
then passed through a series of 16 coulometric electrodes set
5   at 60 mV increments.

Rat Model of Parkinson's

        Female Sprague-Dawley rats received a unilateral
10   injection of 12 μg in 2 μl saline-ascorbate 6-hydroxy-
dopamine (6-OHDA) into the medial forebrain bundle
(coordinates: AP=-4.4; ML=1.1; DV=7.5). Completeness of the
lesion produced was assessed 10 to 20 days postinjection by
either apomorphine (0.1 mg/kg, subcutaneously (s.c.)) or
15   amphetamine (5 mg/kg, s.c.) induced rotational behavior
(Ungerstedt and Arbuthnott, Brain Res. 24:485-493 (1970)).
Prior to transplantation, each animal was tested at least
twice on separate days to establish the baseline rotational
response to apomorphine or amphetamine for each animal.
20   Animals turning at a rate of more than 7 turns per minute
(Schmidt et al., J. Neurochem. 38:737-748 (1982)) were
included in the study (at least 7 contralateral rotations/min
following apomorphine administration and at least 7
ipsilateral rotations/min towards the side of the lesion
25   following amphetamine administration; 19 apomorphine tested,
14 amphetamine tested). The average percent change in the
number of rotations from baseline to post-transplantation was
compared in the 4 experimental groups of animals.

30   ## Grafting of Fibroblasts

        Confluent 10 cm plates of cultured TH-infected or
noninfected fibroblasts were loosened from the plates in PBS
containing 0.05% trypsin and pipetted up in PBS supplemented
35   with 1 mg/ml glucose, 0.1 mg/ml $MgCl_2$ and 0.1 mg/ml $CaCl_2$
(complete PBS) plus 5% rat serum to inactivate the trypsin.
The c lls wer washed twice with compl te PBS using

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centrifugation at 1000 X g and were resuspended in complete
PBS at a density f 80,000 cells per µl. Since graft
placement has b en shown to be crucial f r recovery fr m
rotational asymmetry (Herrera et al., Brain Res. 297:53-61

5   (1984); Dunnett et al., Scand. Suppl. 522:29-37 (1983)),
suspended cells were injected stereotaxically into 2 to 3
separate locations within the rostral (coordinates: AP=1.4;
ML=2.0; DV=3.5-5.5 to AP=2.5; ML=1.5; DV=3.5/4.5) and caudal
areas (AP=0.4; ML=3.0; DV=3.5/4.5) of the denervated caudate.

10   A total of 4 µl were delivered in two equal deposits over a 1
to 2 mm area at each site. Control lesioned animals received
injections of noninfected fibroblasts.

Post-Grafting Behavioral Testing

15
Grafted rats were tested for rotational asymmetry 1 and 2
weeks following fibroblast grafting.

Histological Methods

20
Following the final behavioral test, rats were deeply
anesthetized and perfused with 10% formalin. Brains were
postfixed overnight, placed in 30% sucrose for 48 hrs and then
sectioned (40 µm) on a freezing microtome. Alternate sections

25   were stained for cresyl violet, fibronectin (FB) or TH using a
polyclonal anti-tyrosine hydroxylase antibody (Eugenetech,
NJ). Briefly, the sections were rinsed in Tris-buffered
saline (TBS) solution (pH 7.4) containing 0.25% Triton-X. The
sections were incubated for 24 hrs at 4°C with rabbit

30   polyclonal antibodies to tyrosine hydroxylase diluted 1:600 or
polyclonal anti-fibronectin diluted 1:2000 in TBS containing
0.25% Triton-X and 3% goat serum. After thorough rinsing, the
sections were incubated for 1 hr with biotinylated goat
anti-rabbit IgG (Vectastain) diluted 1:200 in 0.1 M TBS

35   containing 0.25% Triton-X and 1% goat serum, followed by
several rins s in TBS containing 0.25% Trit n-X and 1% goat
s rum. The sections were then incubated for 1 hr at room

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temperature with a c mplex f avidin and biotinylat d
horseradish p roxidase (Vectastain, ABC kit, Vect r Labs,
Burlingame, CA) dilut d 1:100 in 0.1 M TBS c ntaining 0.25%
Triton-X and 1% goat serum, followed by thorough rinses. The
5   peroxidase was visualized by reacting with 0.05%,
3,3-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical
Co., St. Louis, Mo) and 0.05% $NiCl_2$ and 0.01% H_2O_2 in TBS for
15 min at room temperature. Mounted sections were assessed
for size and placement of fibroblast positive grafts.
10

Establishment of a Fibroblast Clone Expressing High Levels of TH

Immortalized, rat fibroblasts (208F) were infected with
15   LThRNL virus produced by the Ψ 2/TH producer cells and 12
G418-resistant clones were established. Table 1 shows the TH
activity of 3 of these 12 G418 resistant clones with the
highest TH activity and the TH activity of the Ψ 2 producer
line. The TH activity of the clones with the highest activity
20   (clones 208F/TH-8 and 208F/TH-11) contained approximately a
quarter of the TH activity of rat striatum. The 208F/TH-8
clone that contained the highest TH activity, was chosen for
further study.

25   Table 1

                TH Activity of Cell and Tissue Extracts

Cell Line	TH Activity*
Ψ2/TH	1.7
208F/TN-8	2.9
208F/TH-11	2.6
208F/TH-9	0.4
208F/CONTROL	0.0
Rat Striatum	9.8

40   *TH activity is expressed in units of
            pmoles DOPA/min/mg protein

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Fibroblasts Expressing TH Produce and Secrete L-dopa.

     Cell extracts from the 208/TH-8 fibroblasts expressing TH
and control 208F fibroblasts were assayed for L-dopa (Table
2). Only 208F/TH-8 cells cultured in media supplemented with
$6MPH_4$ produced L-dopa. Control cells did not contain any
detectable amounts of L-dopa. Dopamine and its metabolites
DOPAC and HVA were below detectable levels in both 208F/TH-8
and 208F control cells.

Table 2

L-dopa Concentration of Cell Extracts and Media

L-dopa Concentration[1]

Cell Clone	Cell Extract		Cell Media	
	no $6MPH_4$[2]	$+6MPH_4$[3]	no $6MPH_4$[2]	$+6MPH_4$[3]
208F/CONTROL	<0.25	<0.25	N.D.[4]	63
208F/TH-8	<0.25	1.38	N.D.	239

1.   L-dopa concentration is expressed in units of
nanograms (ng)/mg protein for cell extracts and in
units of ng/hr/10^6 cells for cell media.

2.   Cell incubated in normal media.

3.   Cells incubated overnight in normal media supple-
mented with 0.1 mM DL-6-Methyl-5,6,7,8-
tetrahydropterin.

4.   N.D. n t d t rmined

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As shown in Table 2, L-dopa was also detected in the
media of the 208F/TH-8 cells: 63 ng/hr per 10^6 c lls in
control 208F media and 239 ng/hr per 10^6 cells in TH-inf cted
media.   There was no detectable levels of L-dopa metabolites

5   contained or released in 208F/TH8 or control fibroblasts.

Histologic Examination of Grafts

       Fibroblast grafts survived intraparenchymal trans-
10   plantation to many areas within denervated caudate.   Surviving
       fibronectin positive grafts were typically moderate to large
       in size regardless of placement (Fig. 18a and b).   Only 4 out
       of 31 grafts were classified as non-surviving based on the
       confinement of fibronectin staining to the syringe tract (Fig.
15   18c and d).   Behavioral data from the rats with nonsurviving
       grafts were excluded from statistical analyses.   TH

       immunoreactivity was not observed in the fibroblasts either _in
       vitro_ or _in vivo_.
20
Effect of Grafts on Rotational Asymmetry

       The number of drug-induced rotations for each individual
       animal were compared before and 2 weeks after transplantation.
25   Rotational scores from rats tested with apomorphine were
       pooled with those from rats tested with amphetamine since no
       difference was seen between these groups.

       Amelioration of rotational asymmetry was dependent on
30   graft placement.   Rats with fibroblast grafts confined to
       caudal areas of the caudate (Fig. 19 Top) (AP=0 to 0.4) had no
       significant changes in rotational behavior.   Rats which had
       surviving TH-infected fibroblasts in rostral caudate striatum
       (AP=1.4 to 2.2) showed an average 33% reduction in
35   drug-induced rotations 2 weeks following transplantation (Fig.
       19 Bottom).

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These results demonstrat that the rat cDNA coding for
the TH g ne can express functional TH enzymic activity when
stably transduced into fibroblasts. Fibroblasts expressing
the TH gene can produce and secrete L-dopa *in vitro*. When
5   these DOPA-producing fibroblasts were implanted into the
rostral caudate region, they substantially and significantly
reduce the rotational asymmetry in the rat model of
Parkinson's. Since control fibroblasts do not produce
significant levels of L-dopa *in vitro* and do not attenuate the
10   rotational asymmetry of these rats, the ability of these
DOPA-producing cells to attenuate these rat's rotational
symmetry must be due solely to the presence of the TH gene
within the cells. These data demonstrated an effect on
rotational behavior for at least two weeks.
15

The effect of the DOPA-producing fibroblasts on
rotational behavior were dependent on placement in the rostral
caudate. Previous data utilizing fetal neuronal grafts into
rats have shown that attenuation of rotational asymmetry is
20   best achieved when the grafts are placed into the rostral
caudate, (Dunnett, *supra*). Since the fibroblasts used cannot
sprout axons, the location of the graft is even more
critically dependant upon proper graft placement.

25   The exact mechanism by which the DOPA-producing
fibroblasts reduce rotational asymmetry remains to be
determined. Presumably, once L-dopa is secreted, there
remains enough caudate DOPA decarboxylase activity, even
within these totally denervated animals (Lloyd et al., *Science*
30   170:1212-1213 (1970); Hornykiewicz, *British Med. J.* 29:172-178
(1973)), to convert L-dopa to dopamine that then modifies
drug-induced rotational behavior. This postulated mechanism
of action of these DOPA-producing cells would be consistent
with the well established efficacy of systemic L-dopa therapy
35   for Parkinson's disease (Calne, *N. Eng. J. Med.* 310:523-524
(1984)). These DOPA-producing fibroblasts ar in effect small
localized pumps f L-dopa.

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The ability, demonstrated in this example, t modify cells t produce L-dopa br adens the search for th ideal type of cell for transplantati n therapy of Parkins n's. Any cell that can be genetically modified to express the TH gene and

5   that can survive long-term in the brain without forming a tumor or causing other damage, may be used. Although these particular immortalized rat fibroblasts have not formed tumors for up to three months, primary cells such as primary fibroblasts or primary glial cells may offer the theoretical

10   advantage of decreased propensity for tumor formation and may be preferred for grafting in certain applications. In addition, the use of the patients own primary cells for an autologous graft would decrease the chance of graft rejection. However, the use of immortalized cells such as the 208F cells

15   used in this example, does offer the advantage of having large amounts of well-characterized cells readily available.

These results demonstrate that a fibroblast can be genetically modified to supply a function normally supplied by

20   a neuron, therefore not requiring the use of fetal tissue for neuronal transplantation. The ability to combine transplantation modalities with gene-transfer presents a powerful method for the treatment of CNS dysfunction. The methods of the present invention may thus be used for

25   treatment of other models of animal and human brain disease.

<u>Example IV</u>

Grafting of Cultured Autologous Primary Skin Fibroblasts

30

As noted above, it may be desirable in certain circumstances to minimize rejection of intracerebral grafts and avoid tumor formation using autologous cells such as primary skin fibroblasts. In this example, primary skin

35   fibroblasts were used to evaluate this cellular population for intracerebral grafting by assessing the growth and survival f th cells <u>in vitr</u> and <u>in vivo</u> and the int grati n f the

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grafted cells int the d n r's striatum.

In these exp riments, the growth rate f primary skin
fibroblasts in culture was measured. This growth pattern was

5   compared with that of immortalized Rat-1 fibroblasts in vitro,
to determine whether growth and cellular characteristics in
culture would predict those following implantation. Variables
that might influence the survival of primary fibroblast grafts
were also examined, including the number of passages the cells

10   can be taken through in culture before implantation and the
density of cells implanted. Introduction of new genetic
material into cultured cells requires several passes to select
and expand the modified populations, making assessment of the
former parameter important. The density of cells implanted

15   was varied to determine an optimal graft size. The
morphological and neurochemical characteristics of the grafts
were also examined, and the cellular interactions between the
autologous fibroblast grafts and host striatum were revealed.

Skin biopsies were taken from the ventral abdominal wall
of twenty-three female Sprague-Dawley rats (200-250 g). Pure
fibroblast cultures from each biopsy were maintained under
standard conditions and fed DMEM containing 10% fetal bovine

5   serum three times a week. The fibroblasts were grown to
confluency and then split 1:2. The growth rate of primary
skin fibroblasts was determined with the aid of a Coulter
Counter (Coulter Electronics Inc). Triplicate samples of
these cells were counted each day, and the growth rate of

10   primary fibroblasts was compared to that of immortalized Rat-1
fibroblasts grown and sampled under the same conditions.

For intracerebral grafting, primary skin fibroblasts from
each animal were taken to either one or four passages (there

15   were approximately five cell divisions between passage one and
four). These cells were then grown to confluency and
harvested at a r sting stat . The cultured fibr blasts were
suspend d in a solution of grafting ph sphate buffered salin

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supplemented with 1 mg/ml of $MgCl_2$ and $CaCl_2$, and 0.1% glucose. The density of the suspensions ranged from 10^4 t 10^5 cells/μl. Th animals were anesthetized with intramuscular injecti ns of ketamine-xylazine (10 mg/kg Ketarar, Parke-Davis, Ann Arbor,

5   MI; 5 mg/kg Rumpun, Hoechst, Frankfurt, Germany; and 6 mg/kg acepromazine maleate, TechAmerica) and placed in a stereotaxic frame. Each animal received implants of cultured skin fibroblasts taken from its own skin biopsies. A total of four cell suspensions (3 μl/site) were injected stereotaxically

10   into the striatum with a Hamilton syringe, two rostrally and two caudally. Following implantation, the animals survived for periods of three or eight weeks.

To assess parameters that may influence the survival of

15   intracerebral primary skin fibroblast grafts, two experiments were designed to address the issues of cell passage (n=12) and cell density (n=9). First, groups of three rats received injections of their own primary skin fibroblasts (10^5 cells/μl) that were taken through one or four passages; these animals

20   were sacrificed and perfused three or eight weeks following surgery. Second, groups of three animals received suspensions at 10^4, 2.5 x 10^4, 5.0 x 10^4 or 10^5 cells/μl; the number of passages (four) and the survival period (eight weeks) were constant.

25

Tissue Preparation and Histology

For light microscopy, the animals were perfused transcardially with a solution of 4% paraformaldehyde in

30   phosphate buffer (pH 7.3). The brains were removed, post-fixed overnight, and stored in 30% sucrose for three days. Coronal sections through the striatum were cut on a freezing microtome at a thickness of 40 μm and collected in cryoprotectant. One series of sections was stained with

35   cresyl violet. The remaining sections were divided into three groups f r the immunohistochemical d tection of fibr n ctin, glial fibrillary acidic protein (GFAP) and reactiv microglia

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and/or macrophages. Th sections were treated initially with
0.6% hydrogen peroxide in Tris buffer (pH 7.4) f r thirty
minut s to block ndogen us peroxidase activity. Secti ns
were rinsed in buffer and then incubated in rabbit anti-human

5   fibronectin immunoglobulin (IgG) (1:2000 dilution) or rabbit
anti-human GFAP IgG, 1:1000 dilution) with 3% normal goat
serum and 0.25% Triton-X in Tris buffer overnight at room
temperature. Next they were rinsed in buffer and incubated in
biotinylated goat anti-rabbit IgG (1:250 dilution) for one

10   hour. After another rinse, they were incubated in avidin-
biotin complex (Vector Laboratories) for one hour and rinsed.
They were then treated with a solution of 0.025%
diaminobenzidine (DAB) tetrahydrochloride, 0.5% nickel
chloride and 0.03% hydrogen peroxide in Tris buffer for five

15   minutes.

Immunohistochemical localization of reactive microglia
and/or macrophages was achieved using the mouse monoclonal
antibody MRC OX-42 (Serotec). Sections were rinsed in buffer,

20   and then incubated with MRC OX-42 (at a concentration of
10 μg/ml) with 3% normal horse serum and 0.25% Triton-X in
Tris buffer overnight at room temperature. They were rinsed
and incubated in biotinylated horse anti-mouse IgG (1:160
dilution) for one hour. The sections were then rinsed,

25   incubated in avidin-biotin-complex, rinsed again, and reacted
in a DAB solution (as above). Following the immunoperoxidase
reaction, all sections were rinsed in Tris buffer and then in
phosphate buffer. They were mounted on chrome alum-gelatin
coated slides, dehydrated through a graded series of ethanols,

30   and coverslipped.

For electron microscopy, animals (n=2, from the cell
density study) were perfused transcardially with a solution of
4% paraformaldehyde and a 0.1% glutaraldehyde in Tris buffer

35   (pH 7.4). The brains were removed and post-fixed for three
hours, after which coronal sections through the striatum wer
cut on an Oxford vibrat m at a thickness f 50 μm and

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coll ct d in Tris buffer. Sections wer th n ither stain d
immunohistochemically for fibron ctin or fix d in 1% buffered
osmium tetroxid for on hour. The latter s cti ns were
rinsed, dehydrated through a graded series of methanols,
5   cleared in propylene oxide, and embedded in a mixture of
Poly/Bed and Araldite. Semithin sections (1-2 μm) were
collected and stained with 1% toluidine blue to assess the
graft. Ultrathin sections were then cut on a Reichert Om U3
ultramicrotome, collected on copper grids and stained with
10 uranyl acetate and lead citrate. The sections were viewed
with a JEOL 100 CXII transmission electron microscope.

Graft Analysis

15   Since fibronectin is not normally detected in the rat
brain, fibroblast grafts were defined by the area stained
immunohistochemically for fibronectin. Sections possessing
immunoreactivity were analyzed with a Cue-2 Image Analysis
System (Olympus Corp). Immunostaining was traced manually
20 (mag. = 4X) and the area of the graft was computed for each
section in a given series. Graft volume (mm^3) was calculated
from this series according to Uylings et al. (J. Neurosci.
Meth. 18:19-37 (1986)), incorporated by reference herein.
Sections stained for cresyl violet, GFAP and MRC OX-42, as
25 well as sections viewed with the electron microscope, were
evaluated qualitatively.

Growth Curves for Rat-1 Fibroblasts and Primary Fibroblasts

30   The growth rate of Rat-1 cells, which are immortalized
fibroblasts that are putatively contact inhibited, was
compared to that of primary skin fibroblasts cultured under
similar conditions. Although the growth curves of both cell
types were similar for the first week, dramatically different
35 growth patterns were observed after ten days (Fig. 20). Rat-1
cells continued to grow once confluency was reached, and
ther fore did not app ar to be contact inhibited. The growth

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of primary skin fibroblasts, n th ther hand, stabilized
 nce the c lls reached confluency. These patterns f
continued growth (Rat-1 fibroblasts) v rsus qui scence
(primary skin fibroblasts) persisted for up to three weeks.

5

Cell Passage

      A parameter that may influence the size of primary
fibroblast grafts in the striatum was the number of in vitro
10   passages the cells were taken through prior to implantation.
For this study, the cell density of grafts was constant at 10^5
cells/μl. Six animals received cell suspensions of
fibroblasts taken through one passage in culture, and another
six received fibroblasts taken through four passages. Three
15   animals from each group were sacrificed and perfused three or
eight weeks after implantation. A 2-factor ANOVA was employed
to assess the difference in volume of grafts at passage one
and four, and after postoperative periods of three and eight
weeks (Fig. 21). Graft volume, determined with fibronectin
20   immunostaining, did not differ as a function of the number of
passages in culture prior to implantation (one versus four).
It was also evident that cultured autologous fibroblasts
implanted into the brain did not create tumors, nor did these
grafts atrophy and die. In fact, the size of the grafts did
25   not differ significantly after three and eight weeks survival
in vivo.

Cell Density

30   Another parameter of graft size examined was the density
of cells implanted into the striatum. Four densities (10^4, 2.5
x 10^4 and 10^5 cells/μl) were assessed, and the volume of the
grafts was determined immunohistochemically for fibronectin.
The fibroblasts for intracerebral grafting were harvested
35   following the fourth passage in culture and the animals were
sacrificed aft r a post-op rativ period of eight weeks. With
a 2-factor ANOVA, significant diff rences in graft volum w r

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observ d among the fours groups; suspension grafts of high
c ll density produc d larg r grafts than grafts f low cell
density (Fig. 22). Thus, a r lationship betw en the volum f
the graft and the density of cells implanted was evident.

<u>Histology</u>

Immunostaining for fibronectin, a group of structurally
related glycoproteins, revealed the size and limits of the
intracerebral grafts. At three weeks, the grafts possessed
several small extensions into the neuropil of the striatum,
whereas those examined at eight weeks appeared more compact
and possessed fewer projections (Fig. 23). Fibronectin
immunostaining, however, did not allow for the resolution of
individual fibroblasts or other cellular components in or near
grafts. From cresyl violet staining, elongated nuclei that
are typical of fibroblasts were observed in the grafts (Figs.
24a, b). Blood vessels were also found through the grafts,
and debris-filled cells were located predominantly at the
center. Astrocytes stained immunohistochemically for GFAP
were prominent in the host striatum surrounding the grafts at
three weeks, yet immunostaining within the grafts was sparse.
GFAP-immunoreactive processes were observed both within and
around the grafts at eight weeks (Figs. 24c, d). Reactive
microglia and/or macrophages visualized with MRC OX-42
immunoreactivity surrounded the grafts at three weeks, but
were less prevalent and more variable at eight weeks (Figs.
24e, f).

<u>Ultrastructure</u>

The structural organization of primary fibroblast
autografts at eight weeks after implantation was examined at
the electron microscope level. The most striking feature of
the grafts was the abundance of fibroblasts and collagen. The
fibroblasts possessed an elliptical nucleus that was ften
condens d; a distinct nucl lus was rarely observ d (Fig. 25a,

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b). Th cytoplasm of th se c lls contain d rough ndoplasmic
reticulum, longat d mitochondria, and s cr tory vesicles. a
paucity of Golgi apparatus was vident. Slend r fibroblast
processes found throughout the grafts also contained rough

5   endoplasmic reticulum and secretory vesicles (Fig. 25c).
Dense bundles of collagen surrounded fibroblast cell bodies
and their processes. Neither fibroblasts nor collagen were
observed in the striatal neuropil. Other cellular components
of the grafts included reactive astrocytic processes and

10 phagocytes. Hypertrophied astrocytic extensions were
observed in the extracellular matrix of the grafts passing
among collagen bundles; the cell bodies of astrocytes were not
found within the fibroblast implants (Fig. 26a). The
morphology of phagocytic cells of the grafts was variable; the

15 cytoplasm possessed few organelles and was usually filled with
electron-dense debris (Fig. 26b). The nucleus of these cells
was irregular and chromatin was often clumped beneath the
nuclear envelope. A small number of lymphocytes was also
observed around and within the grafts at eight weeks after

20 grafting.

Another prominent feature of the primary fibroblast
grafts was the presence of continuous-type capillaries; the
endothelial lining of the lumen was uninterrupted, and

25 fenestrations were not evident (Fig. 27a). A basal lamina
enveloping these vessels was present. Endothelial cells
surrounding the lumen possessed few organelles and a nucleus
with condensed chromatin under the nuclear envelope.
Numerous vesicular invaginations of both the luminal and

30 abluminal plasma membranes of endothelial cells were evident,
as well as clear vesicles within the cytoplasm (Fig. 27b).
Intercellular junctions were observed at sites of close
apposition between endothelial cells (Fig. 27c). Collagen
and/or reactive astrocytes were closely associated with the

35 graft capillaries. These reactive astrocytic profiles
adjacent t capillari s were fill d with filaments, and did
not r semble those endfe t astrocytes that envelope normal

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cerebral vessels (Fig. 27d). Certain capillari s also
possessed sev ral r activ astrocytic process s compl tely
surrounding th v ssel; th perivascular space b twe n the
endothelium and astrocytes usually possessed collagen and was

5   variable in size.

        Primary skin fibroblasts cease to proliferate once they
are confluent, thereby exhibiting contact inhibition in vitro.
As shown herein, following implantation in the striatum, the

10   graft volume of primary skin fibroblast grafts remains
constant between three and eight weeks; thus a propensity for
either tumor formation or death is not a characteristic of
primary skin fibroblasts in vivo. Features of primary
fibroblasts in culture, e.g., contact inhibition, may reflect

15   the growth and survival of the grafts following implantation
into the CNS. Data from the present investigation also reveal
that the density of the cell suspension is another factor that
determines the size of primary skin fibroblast grafts: cell
suspensions of a high density produce larger grafts than those

20   of a low density. The number of cell passages in culture
prior to implantation and the post-operative period, on the
other hand, do not appear to be parameters that influence
graft size.

25   Another advantage of employing primary skin fibroblasts
for grafting is that the likelihood of immune rejection of
autologous cells is lessened. An immunological response
against allografts, especially 208F fibroblasts, may be one
factor that leads to cell death following implantation. In

30   this example, fibroblasts from skin biopsies were grown under
cultured conditions and implanted into the donor's striatum.
Following stereotaxic placements of fibroblasts in the
striatum, the blood-brain barrier is damaged, and several cell
populations, including lymphocytes, enter the wound area.

35   Despite the presence of lymphocytes near the graft, the
immunological reaction against thes autologous cells appears
to be minimal. Ther fore, the histocompatibility of primary

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fibroblasts may also enhance graft survival _in vivo_.

This example provides direct evid nce f r the sustained
survival of primary skin fibroblasts grafted in the adult rat
5   CNS. Ultrastructural analysis reveals that these grafts are
composed primarily of fibroblasts and collagen, in addition to
possessing other cellular populations (e.g., phagocytes,
lymphocytes). It is noteworthy that the morphological
features of grafted fibroblasts after eight weeks in the
10   striatum are similar to those normally found in the skin,
including an elliptical nucleus with condensed chromatin,
elongated mitochondria, and a few Golgi apparatus. The
presence of rough endoplasmic reticulum and secretory vesicles
within the cytoplasm and processes of the fibroblasts strongly
15   indicates active protein production. Further, the abundance
of fibronectin immunostaining and collagen provide evidence
for the synthesis of two distinct cellular products of
fibroblasts within the grafts. Despite collagen production,
the size of the grafts remains relatively constant between
20   three and eight weeks after implantation. One plausible
explanation for this observation is that cultured fibroblasts
are known to produce collagenase (Millis et al., _Exp. Geront._
24:559-575 (1989)); fibroblasts may therefore regulate the
levels of collagen production and degradation within the
25   grafts. From the light and electron microscopic date, it is
concluded that primary skin fibroblasts grafted in the rat
striatum maintain their morphological characteristics, produce
and secrete collagen and fibronectin, and survive up to eight
weeks. Although the total number of surviving cells or the
30   percentage of cells that survive was not determined, a higher
percentage of cells within the grafts at eight weeks following
implantation is unlikely. Trophic substances such as
fibroblast growth factor (FGF) may augment the survival of
primary skin fibroblasts grafted into the rat CNS.
35

Host-to-graft Interactions

An important consideration of cultured autologous skin
fibroblast implants is wheth r these grafts are integrated
into th host nervous system. Aft r implantation of cultured
primary skin fibroblasts into th rat striatum three prominent
5   cellular events were observed that constitute dynamic
interactions between the host and graft. These may be
temporal events and occur as follows: 1) activation and
migration of microglia and/or macrophages to the graft; 2)
neovascularization; and 3) hypertrophy and infiltration of
10   astrocytic processes into the graft. Data from prior
investigations of the initial response to the CNS to
penetrating wounds that compromise the blood-brain barrier may
be extrapolated to the model system described herein. It has
been shown that mononuclear phagocytes are the first cells
15   that respond to wounds within the CNS (Imamoto and Leblond, _J._
Comp. Neurol. 174:255-280 (1977)). Macrophages, microglia and
granulocytes may be shown immunohistochemically using the MRC
OX-42 antibody which recognizes the rat homologue of
complement receptor type 3 of mouse and man (Robinson et al.,
20   _Immunol._ 57:239-247 (1986)). The results presented herein
indicate that cells possessing OX-42 immunoreactivity are
present at the periphery of the graft at three and eight weeks
after implantation. Electron microscopic examination of
tissue at eight weeks confirms the presence of phagocytic
25   cells in the interface zone between the graft and the intact
neuropil, and these cells may be those stained
immunohistochemically for OX-42. Phagocytic cells were also
present throughout the graft, yet OX-42 immunoreactivity was
not evident within the graft itself. Therefore, non-
30   immunoreactive phagocytes may constitute either another
cellular population or macrophages, microglia and granulocytes
that lack the complement receptor. The cells migrate to the
wound site and infiltrate the graft.

35   An important host-to-graft cellular interaction that may
determine the fate of fibroblasts following implantation is
th stablishment f a vascular system within th grafts. The

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establishment of a vascular system within the grafts may
depend in part on angiog nic substances such as fibr blast
growth factor. Production and release of fibr blast growth
factor by grafted skin fibroblasts may promote angiogenesis.

5   Neovascularization within the grafts may also be enhanced by
reactive microglia and macrophages which produce and release
interleukin-1.

Implants of avascular tissue into the CNS are completely

10   dependent on the surrounding host tissue for the formation of
new capillaries. Capillaries within the skin fibroblast
grafts are composed of non-fenestrated endothelial cells that
form a continuous lumen and possess intercellular junctions
(not necessarily tight junctions) at sites of cellular

15   apposition; these vessels are reminiscent of those found in
both connective and nervous tissues; however several features
distinguish graft capillaries from those in the adjacent
striatum. First, endothelial cells within the grafts possess
numerous intracellular vesicles and vesicular invaginations on

20   the plasma membranes, whereas neural capillaries have few
endothelial invaginations on the luminal and abluminal sides.
Second, reactive astrocytes associated with graft vessels are
unlike endfeet astrocytes surrounding neural capillaries, in
that the former profiles are filled with filaments,

25   hypertrophied and do not always envelope the graft
capillaries. Third, the perivascular space between
endothelium and reactive astrocytic profiles of graft
capillaries is variable and often contains intervening
elements, including collagen; neural vessels possess a narrow

30   perivascular space containing basal lamina.

The third interaction observed between the striatal
neuropil and grafts of primary fibroblasts was the
responsiveness of astrocytes to the autografts. Intracerebral

35   grafting usually results in a glial reaction that involves the
migration of reactive astrocytic processes and/or the
f rmation f a glia limitans. Th s studies show that GFAP-

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immun reactive astr cyte perkarya are abundant around the
graft at thre and eight weeks, and immunoreactive processes
are found within the graft at eight weeks. Ultrastructural
data corroborate the latt r observati n, revealing that
5   reactive astrocytic processes are present throughout the
graft, but astrocytic cell bodies remain outside the graft
itself. Astrocytic responses observed in the present study
and others may be due, in part, to the presence of reactive
microglia and macrophages near the wound site; these cells are
10   known to produce and release interleukin-1 in vitro.

These results provide direct evidence for the survival of
cultured primary skin fibroblasts grafted into the CNS of
adult rats. These grafts do not form tumors within the
15   striatum, and possible immunological responses appear minimal
due to the autologous nature of these cells. Moreover,
fibroblast grafts are structurally and functionally integrated
into the host brain. Dynamic cellular interactions between
the host striatum and fibroblast implants indicating the
20   structural and functional integration of the fibroblasts into
the adult rat CNS include the establishment of a vascular
system within the graft, the migration of phagocytic cells,
and the hypertrophy and infiltration into the grafts of
reactive astrocytic processes. Collectively, these features
25   indicate that primary skin fibroblasts are suitable donor cell
candidates for cerebral grafting, and therefore may be
employed as genetically modified cells for gene therapy in
diseased or damaged CNS.

30   Example V
Grafting of Primary Fibroblasts Producing L-dopa

This example examines the long term survival of
genetically modified primary fibroblasts following grafting
35   into the brain. Extending the above experiments, the rat TH
gene was inserted into primary fibroblasts obtained from a
skin bi psy from Fischer rats. Th survival of TH-containing

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primary fibroblasts was then examin d for a 2 month peri d
following implantation into the brain f Fischer rats with a
prior 6-OHDA l sion f th nigrostriatal pathway. In
addition, both the presence f th TH transg ne and transgene
5   product within the implanted fibroblasts were examined.
Finally, prolonged functioning of the genetically modified
fibroblasts _in vivo_ was explored through assessment of
rotational behavior of the implanted rats every 2 weeks for an
8 week period after grafting.

10

Preparation of Retroviral Vector

Preparation of the vector used in the present
experiments, pLThRNL, was as described above in Example III
15   (see Figures 16 and 17).

Fibroblast preparation

Primary fibroblasts were obtained from abdominal skin
20   biopsies of inbred Fischer 344 rats, according to the protocol
of Sly and Grubb (_in_ "Methods in Enzymology", Vol. LVIII,
Colowick and Kaplan, Eds., Academic Press, New York, pp. 444-
450 (1979)). Briefly, rats were anesthetized with a mixture
of ketamine, aceptomazine and rompun. The abdomen was shaved
25   and scrubbed thoroughly with alcohol. An area of
approximately 1-2 cm^2 of skin was removed, rinsed briefly in
alcohol and immediately placed in a vial containing sterile
DMEM/S. Biopsies were typically transferred to coverslips
within 2 hours of collection and grown in DMEM/S for 21 days.
30   Cells were then infected with ecotropic LThRNL or BAG virus
(Price et al. _Proc., Natl. Acad. Sci. USA_ 84:156-160 (1987))
at a multiplicity of infection of 10 in the presence of
Polybrene (4 μg/ml). G418-resistant cells were grown to
confluency and tested for expression of TH activity by a
35   decarboxylase-coupled assay (Iuvone, _J. Neurochem_ 43:1359-1368
(1984)). A subclone expressing TH activity and TH
immunor activity _in vitro_ was selected for implantation

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(FF2/TH). Fibr blasts infected with the BAG virus and expressing β-galact sidase (βGal) activity _in vitro_ were used as control cells (FF2/βGal).

5   ## In vitro Biochemical Analyses

        The production and release of L-dopa and its metabolites from the infected fibroblasts were assessed after growing cells for 24 hours in DMEM/S supplemented with 100 μm
10   tetrahydrobiopterin, the active cofactor for TH. Conditioned media and cells were collected, adjusted to 0.1 M perchloric acid (PCA) and 0.05 M EDTA, and centrifuged at 10,000 X g for 15 min at 4°C to remove precipitated material. Samples were analyzed for the presence of catecholamines and catecholamine
15   metabolites as described above in Example II by injecting the PCA extracts onto a coulometric electrode array, gradient liquid chromatography system (CEAS model 55-0650, ESA, Bedfore, MA) equipped with 16 electrochemical sensors (Matson et al., _Clin. Chem._ 30:1477-1488 (1984); Langlais et al., _in_
20   "Monitoring Neurotransmitter Release During Behavior", Fillenz, et al., eds., Horwood, Chichester, U.K., pp. 224-232 (1986); and Matson et al., _Life Sci._ 41:905-908 (1987)).

        ## Lesions
25

        The dopaminergic nigrostriatal pathway was unilaterally destroyed with the neurotoxin 6-hydroxydopamine (6-OHDA). The 6-OHDA was dissolved in saline at a concentration of 6 μg/μl and supplemented with 0.1% ascorbic acid to retard oxidation.
30   Female Fischer 344 rats (130-160 g) were anesthetized as above and placed in a stereotaxic frame. A 2 μl injection of 6-OHDA was delivered at 1 μl/min to the left medial forebrain bundle (AP=-4.4 mm, ML=1.1 mm, DV=7.5 mm; according to the atlas of Paxinos and Watson, 1986, "The Rat Brain in Stereotaxic
35   Coordinates", San Diego, Academic Press, (1986)). Following the injection, the syringe was raised 2 mm and allowed to rest an ther 2 minutes to permit diffusi n f the neur toxin.

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After a 10 t 14 day recovery period, lesioned rats were
tested with ap m rphine t assess the extent of th lesi n.

To assess the contribution f n n-specific striatal
5   damage to changes in rotational behavior the procedures
described above in Example III were employed using 6-OHDA
lesioned rats subjected to unilateral kainic acid (KA) lesion
of the striatum. For this experiment, female Sprague-Dawley
rats were anesthetized and lesioned as above with 6-OHDA.
10   Rats which displayed greater than 7 rotations per minute to
apomorphine (0.05 mg/kg) were selected for KA lesion. Kainic
acid was dissolved in saline at a concentration of 5 μg/μl and
injected into the striatum (AP=0.3 mm, ML=2 mm, DV=4 mm) of
anesthetized rats at varying doses. Three rats received
15   1.0 μg, 3 rats received 2.0 μg, 3 rats received 4.0 μg and 2
rats received 5.0 μg.. The KA was delivered at a rate of 0.1
μl/min, and the syringe was then slowly lifted 2 mm and left
in place for an additional 2 minutes to allow diffusion of the
neurotoxin. Rats were administered diazepam (2 mg/kg)
20   immediately following the injection to block seizures which
may have resulted from the use of the KA.

Behavioral Testing

25   The sensitivity of postsynaptic dopamine receptors within
the denervated striatum was assessed 7-10 days after 6-OHDA
lesion with apomorphine (0.1 mg/kg) administration as
described above in Example III. Rats were placed in automated
rotometer bowls and total rotations were collected every 10
30   minutes for a period of 60 minutes. After 2 apomorphine
tests, rats which displayed at least 7 rotations/minute for 60
minutes were selected for further study. Rats were tested
with apomorphine at least 3 times or until the number of
rotations did not change from the previous testing session
35   (Bevan, Neurosci. Letter 35:185-189 (1983); Castro et al.,
Castro et al., Psychopharm. 85:333-339 (1985)). A period of 4-
10 days elapsed b tween each apom rphine test. After

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grafting, rats were tested with ap m rphin (0.1 mg/kg) every
2 we ks for 8 weeks. Rats with KA lesions were tested with
apom rphine (0.05 mg/kg) 2, 4 and 6 weeks after lesioning.
For statistical comparis ns, the n t rotati ns/minute over a
5   60-minute time period were determined following grafting and
compared to pre-transplantation values using the Least Squares
Difference Test.

Grafting

10

        Fibroblasts were prepared for grafting as described above
in Example II. Briefly, cells were removed from tissue
culture plates with 0.05% trypsin and 1 mM EDTA in Dulbecco's
PBS, and suspended in PBS supplemented with $MgCl_2$, $CaCl_2$, 0.1%
15   glucose (complete PBS) and 5% rat serum. Cells were collected
by centrifugation, washed twice with complete PBS, and
resuspended in complete PBS at a density of 100,000 cells/μl.

        Fifteen anesthetized rats received a total of 10 μl of
20   either FF2/TH (N=10) or FF2/βGal (N=5) fibroblasts injected
into two sites within the striatum (AP=0.3 mm, ML=2.0 mm,
DV=4.5 mm, AP= 1.5 mm, ML=2.0 mm, DV=4.5 mm). At each site,
2.5 μl of cells were dispensed, the syringe was raised 1 mm,
and an additional 2.5 μl of cells were ejected from the
25   cannula. Cells were delivered at a rate of 1 μl/min.   After
the most dorsal injection, the syringe was left in place for 2
minutes to allow diffusion of the cells. An additional 5
lesioned rats, which were not grafted, served as untreated
controls.

30

Anatomical Procedures

        For in vitro immunohistochemical analyses, infected
fibroblasts were fixed onto slides with 4% paraformaldehyde,
35   permeabilized with 0.2% triton X-100 and incubated overnight
with a monoclonal antibody to TH (Boehringer Mannehim
Bi chemicals). The stain was developed by the avidin-biotin

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m thod (V ct r Laboratories, Elite Kit) with nickel-
intensified 3,3'-diethylaminobenzidene (DAB) as the chromagen.

5   For _in vivo_ analyses, implanted fibroblasts were examined
for TH- .and fibronectin-immunoreactivity 10 weeks after
grafting. Rats were deeply anesthetized and perfused with 4%
paraformaldehyde following a brief saline rinse. Brains were
removed and placed in the same fixative overnight and then
transferred to 30% sucrose until they equilibrated. Frozen
10   sections were cut on a sliding microtome (40 μm) and collected
in cryoprotectant for storage until processing. Free floating
sections were incubated overnight with either an antibody to
the fibroblast marker (Organon Technica), or with the
monoclonal antibody to TH. Fibronectin staining was developed
15   with avidin-biotin followed by nickle-intensified DAB. TH
labeled sections were incubated with a secondary antibody to
the mouse antigen conjugated to rhodamine for fluorescence
microscopy (Jackson ImmunoResearch Labs., Inc., Bar Harbor,
Maine). Some sections were also processed with antibodies OX-
20   42 (Serotec) or ED-1 (Chemicon) for identification of
microglia or monocyte/macrophages. Sections were incubated
with the OX-42 antibody for 48 hours and the ED-1 antibody for
24 hours at 4°C. The following day, OX-42 was developed with
avidin-biotin with nickle-intensified DAB while the ED-1
25   labeled sections were incubated with the rhodamine-labeled
secondary antibody as described above.

In situ Hybridization

30   In situ hybridization for TH mRNA was performed using
[^{35}S] labelled probes as described by Higgins et al., _Proc._
Natl. Acad. Sci. (USA) 85:1297-1301 (1988), incorporated by
reference herein. A 63 base pair template for the TH probe
was generated by digestion of the plasmid pSP65 containing a
35   311 nucleotide cDNA complementary to rat TH mRNA (provided by
Dr. Dona Chikaraishi, Tufts Medical School, Boston, MA) with
Ava II, and transcribed by the SP6 p lymerase. Slide-m unted

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sections were fixed for 1 minute with 4% paraformaldehyde,
dig st d with prot inase K for 5 minutes and th n incubated in
triethan lamine (0.1 M, 0.9% NaCl and 0.1% acetic anhydride)
for 10 minutes at room temperature. S cti ns were

5   subsequently dehydrated in graded alcohols followed by
chloroform for 5 minutes at room temperature, returned to 100%
ethanol for 2 minutes and then 95% ethanol for another 2
minutes prior to air drying. Dehydrated sections were
prehybridized for 1-3 hours in buffer containing 50%

10   formamide, 0.75 M NaCl, 20 mM Pipes (pH 6.8), 10 mM EDTA, 250
mM DTT, 5X Denhart's solution (0.02% BSA, 0.02% Ficoll, 0.02%
polyvinylpyrrolidone), 0.2% SDS, 10% dextran sulfate, and 500
μg/ml denatured yeast tRNA. Following prehybridization,
excess buffer was removed and sections were hybridized at 55°C

15   for 18 hours with 10-15 ng of labeled probe in a total volume
of 75 μl of hybridization buffer. Slides were thoroughly
rinsed following the hybridization in solutions containing
0.6M NaCl, 0.06 M Na citrate with or without RNase. Following
posthybridization rinsing, the sections were air-dried and

20   exposed to X-ray film (Kodak XAR) for 24 hours. The sections
were then dipped in Kodak NTB-2 emulsion, exposed for 4 days
(at 4°C), and developed.

When the bulk population of TH-expressing fibroblasts was
25   stained for TH-immunoreactivity, differing intensities of TH-
labeling were observed. To try to isolate a clone that stably
expressed high levels of TH, the bulk population of TH-
expressing cells was subcloned. The clone selected, FF2/TH,
exhibited a relatively homogeneous TH-immunoreactivity (Fig.
30   28). This clone was similar in gross morphological appearance
to uninfected fibroblasts or fibroblasts infected with the
vector carrying βGal. Cells were generally elongated with
multiple processes extending from the soma. Cells expressing
βGal did not display TH-immunoreactivity in vitro.
35

In Vitro Analyses

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Primary fibroblasts infect d with vectors carrying gen s
f r TH or βGal (FF2/βGal) wer examined for TH activity using
a d carb xylase-coupled assay. TH activity was nly found in
FF2/TH cells, at a lev l f 1.1±0.4 pmole L-dopa/minute/mg
5   protein. This level of TH within the FF2/TH primary
fibroblasts was comparable to that seen previously for
immortalized 208F fibroblasts infected with the same LThRNL
vector (see Example III above).

10   Infected fibroblasts were assayed for the production and
release of L-dopa and other catecholamines 24 hours after
exposure to 100 μM BH_4, the natural cofactor for TH. Medium
collected from cells containing the TH gene was found to
contain 17.2 μg L-dopa/10^6 cells. Intracellular quantities of
15   L-dopa, as assessed through analysis of the FF2/TH cell
pellet, were 0.2 μg L-dopa/10^6 cells. Control FF2/βGal cells
did not contain or release L-dopa. There were no detectable
metabolites of L-dopa, such as dopamine, 3,4-
dihydroxyphenylacetic acid or homovanillic acid, in samples
20   collected from the media or cell pellet of either FF2/TH or
FF2/βGal cells.

Behavioral Analyses-fibroblast implanted rats

25   All 6-OHDA lesioned rats implanted with FF2/TH or
FF2/βGal cells were eating and drinking normally within 24
hours of the transplantation surgery and showed stable or
slightly increasing body weight during the 2-month post-
grafting test period. There was no obvious change in
30   spontaneous behavior of the grafted rats; grooming and
locomotor behaviors were comparable to those displayed by the
lesioned control rats that were not grafted.

To assess the production and release of L-dopa from the
35   implanted cells in vivo, a behavioral test was conducted.
Rats were administered apomorphine, which results in
activati n f receptors in the dopamine-depl t d striatum.

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This drug-induced stimulati n causes the rats to walk in a
circular or rotational pattern which can b quantifi d
(Ungerstedt and Arbuthnott, Brain Res. 24:485-493 (1970)).
F llowing grafting, average rotations per minute over a 60

5   minute period following apomorphine administration were
determined for grafted and control non-grafted rats and
compared to pretransplantation rotations for statistical
analyses. Control animals showed a slightly increased level
of rotations following grafting (Fig. 29, control) which was

10   not statistically different from that observed prior to
grafting (p>0.05). The apomorphine-induced rotations of rats
implanted with βGal-containing cells were also not different
from pregrafting rotations through the 2-month post-grafting
time period (p>0.05; Fig. 29, FF2/βGal). In contrast, FF2/TH

15   rats displayed a significant decrease in rotational behavior 2
weeks after grafting, which persisted through 8 weeks post-
implantation (Fig. 29, FF2/TH). The initial 65% decrease in
rotations of FF2/TH rats, from 11.7 ± 3.0 to 4.1 ± 2.9 (mean ±
standard error of the mean), narrowed to a plateau of

20   approximately 30% below pre-grafting levels between 6 and 8
weeks post-grafting.

Anatomical Analyses

25   Fibroblast grafts were present within the brains of all
of the implanted rats 10 weeks post-implantation. However,
grafts from 2 animals were lost during processing. In
general, the placement of FF2/TH and FF2/βGal grafts were
found to be similar, both extended along comparable rostral-

30   caudal and medio-lateral domains. Typically, however, FF2/TH
grafts were larger than FF2/βGal grafts. Within the FF2/TH
group, there was no correlation between the size of the grafts
and reduction of rotational behavior.

35   All grafts contained elongated fibroblast-like cells
interspersed among an abundance of collagen fibers. In
addition to typical fibroblasts, large yell w cells,

apparently filled with hemasiderin, were seen within the core
f the grafts. As th se cells wer   ften filled with debris,
secti ns thr ugh the graft wer stained with OX-42, and ED-1,
mark rs specific for microglia and mon cyte/macrophages.

5   However, while immunoreactive macrophages were found on the
periphery of the grafts, the large debris-filled core cells
did not label with OX-42 or ED-1.

        To assess whether the TH transgene continued to be
10   expressed within the implanted fibroblasts, sections through
the graft were processed for _in situ_ hybridization to TH mRNA.
Positive hybridization for TH mRNA was only found to be
present over cells in the grafts containing FF2/TH fibroblasts
(Fig. 30a). In both FF2/TH and FF2/βGal grafts (Fig. 30b,
15   control), areas of the graft which were predominantly composed
of collagen fibers showed a grain density which was
significantly less than the surrounding background. The
morphology of the cells displaying increased grain density was
varied, with many exhibiting the elongated shape
20   characteristic of fibroblasts (Figure 31c). However, some
cells with positive TH hybridization were observed to be large
and rounded, often filled with hemasiderin and granulated
particles. There were no cells present within βGal grafts
which displayed specific hybridization to TH mRNA (Figure
25   31d).

        To determine if the transgene products were synthesized
and present within the fibroblasts, grafts were examined for
TH-immunoreactivity and βGal histochemistry. Within the
30   FF2/TH grafts, TH-labeled cells with the elongated shape
typical of fibroblasts were observed to be scattered among the
collagen fibers (Fig. 32a). Autofluorescent material was
present within the grafts but was readily discernible form the
rhodamine-specific labeling (emission 582 nm) by the shape of
35   the material and its broad fluorescence through 528 nm (Fig.
32a and 32b, asterisks). There was a heterogeneity in the
intensity of th TH labeling, as observ d _in vitro_.

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Autofluorescent cells, but n discernible TH-immunoreactive
fibroblasts, were present within FF2/βGal grafts.

   Sections stained f r βGal histochemistry revealed diffuse
5 cytoplasmic labelling of cells within the FF2/βGal grafts
   (Fig. 31a). FF2/βGal staining was observed in some of the
   large rounded cells within the core of the graft and in cells
   with the elongated shape typical of fibroblasts (Fig. 31b).
   βGal reactivity within FF2/TH grafts was confined to a small
10 number of rounded cells with the blue granular staining
   previously described for endogenous macrophages (Shimohama et
   al., Mol. Brain Res. 5:271-278 (1989)).

   These results confirm that primary fibroblasts obtained
15 from a skin biopsy can be grown and genetically manipulated in
   culture to express a transgene. Evidence that the inserted TH
   gene product was synthesized and biologically active in vitro
   was provided by histological and biochemical measures. Cells
   containing the TH gene showed positive immunoreactivity for TH
20 in vitro. And, in the presence of the pterin cofactor for TH,
   tyrosine present in the cell culture medium was converted to
   L-dopa through TH enzymatic activity.

   The fundamental advantage of primary fibroblast cells for
25 genetic manipulation is the potential for using these altered
   cells as donor material for autotransplantation and thus
   minimizing problems with immunological responses from the
   host. The primary fibroblasts used in the present study were
   not implanted within the same donor animal; rather,
30 genetically inbred Fischer 344 rats were used as both donors
   of the fibroblasts and recipients of the altered cells. This
   isograft approach was selected as it simplified the
   maintenance of the cells in vitro, while still providing
   conditions which approximated those of autotransplantation.
35 Following iso-implantation into the brain of rats with 6-OHDA
   lesions, it was found that primary fibroblasts containing
   genes for either βGal or TH survived for at least 10 weeks.

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Further, implanted fibroblasts continued t   xpress the βGal
and TH transgenes through th 2-monthly post-grafting survival
period. Evidence that the TH-containing cells continued to
produce and secrete L-dopa _in vivo_ was provided by a
5   behavioral measure: a significant decrease in rotational
asymmetry was observed only for those rats with grafts of TH-
containing fibroblasts.

Fibroblast survival within the brain

10

When examined at 2 months, FF2/TH grafts were observed to
be larger than FF2/βGal grafts. The reason for this
difference is not known. There are many factors which may
contribute to the survival of primary fibroblasts within the
15   brain, including the state of the cell prior to implantation
and the time that cells remain in culture prior to grafting.
The FF2/TH cells used in the present study were a subclone of
a TH-expressing fibroblast, while the FF2/βGal cells were a
bulk population of βGal-expressing fibroblasts. The
20   subcloning procedure which caused the FF2/TH cells to undergo
more doublings than the bulk FF2/βGal cells may have
influenced the ability of the fibroblasts to survive _in vivo_.
Through early passages there does not appear to be a
correlation between the size of a fibroblast graft and the
25   number of cell divisions in culture prior to grafting as
revealed in Example IV. Fibroblasts carried longer in
culture, as were the FF2 cells used in the present study, have
been seen to survive well within the brain over 6 months with
essentially no change in graft size or composition after 4-6
30   weeks.

Additional factors which may affect fibroblast survival
within the brain include the accessibility of implanted cells
to an adequate nutrient supply. Vascular processes were
35   evident within FF2/TH grafts and less obvious within the
smaller FF2/βGal grafts. The time course for the development
of a vascular supply to th grafts may als affect the long-

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t rm survival f the fibr blasts. Grafts c ntaining ther
cell types, such as fetal neur ns, have been f und t be
d p ndent n rapid vascularizati n f r survival p st-
implantation (Stenevi et al., Brain Res. 114:1-20 (1976)).

5   Whether blood vessels within the grafts establish a barrier to
macromolecules, as typical of brain vasculature, or remain
leaky, as may be typical of grafts of peripheral origin
(Rosenstein and Brightman, J. Comp. Neurol 250:339-351 (1986),
may also influence the survival of implanted fibroblasts. A

10   rupture in brain vasculature following grafting has been
suggested to provide a "window" into the brain for factors
which are not typically available to cells within the brain
(Rosenstein and Brightman, supra; Sandberg et al., Exp.
Neurol. 102:149-152 (1988)). Such factors may be beneficial

15   to cells which have been maintained in a serum-enriched
culture medium (Gibbs et al., Brain Res. 382:409-415 (1986);
Ezerman, Brain Res. 469:253-261 (1988)). Conversely a
vascular portal may place the grafted cells in a position
vulnerable to attack from circulating macrophages.

20

Composition of the graft

Although implanted cells were found to survive through a
2-month period, all grafts were characterized by a core of

25   large, hemasiderin and debris-filled cells which initially
suggested a sustained macrophage infiltration. However, while
a limited number of ED-1-positive macrophages were observed
scattered around the periphery of some grafts, cells within
the core of the graft were not immunoreactive with either ED-1

30   or OX-42, markers which are specific for microglia and
monocytes/macrophages. Further, when grafts were stained for
βGal, an enzyme which had previously been shown to exhibit a
distinct granular appearance within endogenous macrophages
(Shimohama et al., supra. 1989), none of the large core cells

35   within either the FF2/TH or FF2/βGal grafts displayed granular
staining. However, some of the core cells within the FF2/βGal
grafts did display a diffuse and cytoplasmic βGal reactivity,

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labelling which has previously been shown to be characteristic
f immortalized fibroblasts which contain the βGal transgene
(Shim hama t al., supra. 1989). Finally, s me f the large
core cells within the FF2/TH grafts display d positive

5   hybridization to TH mRNA. These observations strongly
suggested that the phagocytic cells within the core of the
fibroblast grafts were derived from the original donor cell
suspension.

10   <u>Mechanisms of Graft Function</u>

The possibility that the fibroblast implantation produced
an artifactual "recovery" in behavior due to damage of
striatal tissue and an associated loss of postsynaptic

15   dopamine receptors was considered. However, there were two
pieces of evidence which suggested that this was unlikely.
First, FF2/βGal rats implanted with the same volume and number
of cells as the FF2/TH rats did not show a significant change
in rotational behavior following grafting. Second,

20   destruction of intrinsic striatal neurons and receptors with
kainic acid resulted in a post-damage rotational profile which
was distinctly different from that observed for FF2/TH rats.
Rats with the smallest KA lesions, or those which affected a
much smaller area than that encompassed by the grafts, showed

25   a steady decrease in rotations from 19% at 2 weeks to more
than 50% by 6 weeks post-damage, consistent with the known
degenerative changes which occur within the striatum following
KA administration (Coyle and Schwarcz, <u>Nature</u> 263:244-246
(1976)). In contrast, the most pronounced decrease in

30   rotations observed for FF2/TH grafted rats occurred 2 weeks
after grafting (65%), followed by a gradual increase to a
stable, but higher, number of rotations between 6-8 weeks (30%
below baseline). This apparent decrease in graft efficacy
between 2 and 8 weeks may result from several different

35   mechanisms. First, some of the grafted cells may be lost
during the initial weeks after grafting. In other studies,
fibroblast grafts have be n f und t shrink in size within the

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brain through 3 weeks following implantation. After this
time, a stabl graft size is attain d which persists f r at
least 6 months. A second reas n f r reduced graft efficacy at
long p st-grafting intervals may inv lve an alteration in the
5   metabolic properties of the fibroblasts. Established
fibroblasts may assume properties of quiescent cells with
reduced metabolic activity (Dean et al., _J. Biol. Chem._
261:9161-9166 (1986); Rao and Church _Exp. Cell Res._ 178:449-
456 (1988)), and decreased production of L-dopa. Finally,
10   graft effectiveness may be compromised by genetic events such
as deletion or rearrangement of the TH cDNA inserted in the
fibroblasts, or epigenetic shut-off of transcription from the
LTR promoter driving transcription of the TH cDNA. Such
occurrences have been documented in other retroviral vectors
15   (Xu et al., _supra_, 1989).

One final possibility which could account for a reduction
in apomorphine rotations of the FF2/TH-implanted rats is that
the grafts may have disrupted cortico-striatal interactions
20   through non-specific damage to cortical tissues. Such damage
is reported to reduce rotational behavior of 6-OHDA-lesioned
rats (Freed and Canon-Spoor, _Behav. Brain Res._ 32:279-288
(1989)). However, in our previous work with immortalized Rat-
1 fibroblasts which exhibited uncontrolled growth within the
25   brain, extensive damage restricted to cortical tissues was not
associated with a drop in apomorphine-induced rotational
behavior. As the grafts examined in the present study
exhibited much more limited graft extension into cortical
tissue than was seen for Rat-1 grafts, it seems unlikely that
30
the decreased rotations of FF2/TH rats reflected non-specific
damage to cortico-striatal pathways.

Currently, L-dopa is the most effective treatment of the
35   symptoms of Parkinson's Disease (PD). However, prolonged
systemic administration of L-dopa results in undesired side
effects, such as dyskin sias and an "on-off" phenomena

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(Marsden and Parks, Lancet 7:292-296 (1976)). It has been
suggested that some f these problems result fr m fluctuating
levels f L-dopa in the plasma f llowing ral ingestion of L-
dopa, and may be alleviated by continuous infusion of L-dopa

5   (Nutt et al., New Eng. J. Med. 310:483-488 (1984); Quinn et
al., Neurology 34:1131-1136 (1984)). Intracerebral grafts of
cells genetically modified to produce L-dopa or dopamine may
provide an alternative for controlling the symptoms of PD, and
minimizing untoward side effects of extended L-dopa

10   administration, by providing a continuous, local infusion of
dopamine directly to appropriate target areas within the
brain.

        These results demonstrate that primary fibroblasts
15   genetically modified to express a transgene provide viable
donor cells for intracerebral implantation and a useful
strategy for the treatment of human neurological disease such
as PD.

20   Example VI

                Ability of Aged Fibroblasts to Serve as Donor Cells

                This example demonstrates that aged human fibroblasts may
25   be useful as donor cells for intracerebral grafting.

30   Retroviral Construct for Human Nerve Growth Factor ((NGF)

                A molony murine leukemia virus vector (MoMLV) containing
the human NGF cDNA (obtained from Syntex Research, Palo Alto,
CA), pLhNRNL was used. This vector was prepared by inserting

35   the NGF cDNA into plasmid pLChRNL (see Example VII, Fig. 34)
in place of the dChAT gene. The 5' long terminal repeat (LTR)
drives the expressi n f the human NGF (hNGF) cDNA. Internal

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R us sarcoma virus LTR promoter drives the xpressi n of the
selectable bacterial n omycin resistant gene (ne ^{R})).

Production of Viral Stock

5

Production of infectious virus stock was accomplished by
first transfecting the amphotropic cell line PA317 as
described by Miller and Buttimore, Mol. Cell. Biol. 6:2895-
2902 (1986) (provided by Dr. Miller, Fred Hutchinson Cancer

10   Research Center, Seattle, WA) with the plasmid DNA by calcium
phosphate co-precipitation. Conditioned media from these
cells were collected, filtered and used to infect ecotropic
helper cell line psi (ψ)2 as described by Mann et al. Cell
33:153-159 (1983). The conditioned media from these cells were

15   then used to infect amphotropic helper cell PA317. Producer
cells from this line were selected for their expression of the
neo^R gene by growing the cells in culture medium containing 400
µg/ml of the neomycin analog G418 ((Gibco/BRL, Gaithersburg,
MD). Virus from the PA317 clones, producing the highest titer

20   (approximately 4 X 10^4 c.f.u./ml) were used to infect various
cell lines as described below.

Infection of Cells

25   Primary fibroblasts, obtained from skin biopsy and
established as cell lines, were infected with amphotropic
retrovirus. Young rat (approximately 3 months) primary
fibroblasts were infected with ecotropic virus expressing
mouse NGF or amphotropic virus expressing human NGF. Primary

30   skin fibroblasts from aged humans (male 66 years of age with
Alzheimer's and male 69 years of age without Alzheimer's) were
obtained by skin biopsy from Dr. R. Katzman, University of
California, San Diego, CA. Young (neonatal) human skin
fibroblasts were obtained from the Core Tissue Culture

35   Facility, University of California, San Diego, CA. Rhesus
monkey skin fibroblasts (10 year old male and 11 year old
female) were obtained by skin bi psy from the Primate Center,

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University of Calif rnia, Davis, CA.

   Fibroblasts, gr wn t 70 t 80% confluency, were
incubated ov rnight with 10 ml f m dium containing the
5 retroviruses and 4 μg/ml of polybrene. The infection was
performed for 3 days in a row. Subsequently, the medium was
aspirated and replaced with fresh medium containing 400 μg/ml
of G418. After infection, the cells were continually
maintained in G418 supplemented culture medium.

10

Assay of NGF Activity

   NGF production and secretion by the infected fibroblasts
were assayed by a two site ELISA assay using anti-β NGF
15 antibody (Boehringer-Mannheim, Germany) as described above in
Example II. NGF activity was measured in the medium on the
days indicated in Fig. 33. Fig. 33 shows the amount of NGF
secreted at different confluency from fibroblasts of different
origin expressing either human NGF (hNGF) or mouse NGF (rat
20 fibroblasts). In all cases the production of NGF increased or
at least remained constant (for monkey cells) during
confluency. There was almost no difference in production of
NGF by young or aged human fibroblasts during log phase or
confluency.

25

   These results demonstrate that nonhuman primate (e.g.
monkeys) models of human disease can be used to test the
efficacy of grafting genetically modified transgenes. In
addition, these results show that aged human fibroblasts may
30 be successfully infected with vectors carrying transgenes, and
suggest that aged human fibroblasts may be used as donor
cells.

Example VII

35

Regulation of the Release of Transgene Product
from Don r Cells

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This example illustrates the regulation f s cretion f a transgene product, acetylch line (ACh), fr m transfected cells using ch line.

Cell Culture

Rat-1 fibroblasts were grown in DMEM supplemented with 0.1% glutamine and 10% FBS. Cultures were incubated in a 10% CO_2 atmosphere at 37°C.

Retroviral Construct

A Moloney murine leukemia viral vector (MoMLV) containing the Drosophila ChAT (dChAT) cDNA (obtained from Dr. P. Salvaterra, Duarte, CA), pLChRNL, was constructed as shown in Fig. 34. A 2519 basepair (bp) HindIII/EcoRI fragment, containing the dChAT cDNA from the plasmid pCha-2 (Itoh et al., Proc. Natl. Acad. Sci. USA 83:4081-4085 (1986)), a 377 bp EcoRI/BamHI fragment from pBR322, and a 6500 bp fragment of the retroviral construct pLLRNL (Xu et al, Virology 171:331-341 (1989)) were ligated by standard procedures (see Sambrook et al., In: Molecular Cloning, A Laboratory Manual, ed. C. Nolan, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)). A 5' LTR within pLLRNL drives the expression of the dChAT gene. Similarly, an internal RSV promoter drives the expression of the selectable bacterial neomycin resistance gene (neo[R]).

Infection

Production of infectious vector stock was accomplished by first transfecting the amphotropic helper cell line PA317 (Miller and Buttimore, Mol. Cell. Biol. 6:2895-2902 (1986)) with the plasmid DNA. Medium conditioned by these cells was collected, filtered and used to infect the ecotropic helper cell line Ψ-2 (Mann et al., Cell 33:153-159 (1983)). Producer cells f rm this lin w re selected f r th ir expressi n of n [R]

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by growing th c lls in culture medium containing 400 µg/ml of
the neomycin analog G418 (Gibc). Th tw G418-r sistant
col nies, from a t tal f 16, that expressed the highest level
of ChAT activity and the highest titer of virus (appr ximately
10⁴ c.f.u./ml) were used to infect Rat-1 cells.

Rat-1 fibroblasts, grown to confluence on a 10 cm tissue
culture plate, were split 1:5 and allowed to grow for one day
before they were incubated with 5 ml of medium containing the
retroviruses and 4 µg/ml of polybrene. Cells were incubated
overnight in this medium. Subsequently, the medium was
aspirated and replaced with fresh medium containing 400 µg/ml
of G418. After infection, the cells were continually
maintained in G418 supplemented culture medium.

Determination of ChAT Activity

The method used to determine ChAT activity is a
modification of the procedure described by Fonnum, in the _J._
Neurochem. 24:407-409 (1975), incorporated by reference
herein. Fibroblasts were seeded into 35 mm tissue culture
dishes and allowed to reach 70-80% confluence. Cells were
subsequently washed with PBS and collected by scraping the
cells in 0.5 ml homogenization buffer (0.87 mM EDTA solution
containing 0.1% Triton X-100). The resuspension was
transferred into a 1.5 ml Eppendorf tube and sonicated (3X,
for 10 sec). Ten microliters of the sonicated homogenate was
placed into another Eppendorf tube. Ten microliters of
radioactive solution (0.2 mM ^{14}C acetyl Coenzyme A, 1 mg/ml
BSA, 0.2 mM eserine salicylate, 4 mM choline chloride, 18 mM
EDTA, 0.6 mM NaCl, and 0.1 mM NaH₂PO₄) was added. This mixture
was incubated at 37°C for 5 min. The reaction was stopped by
adding 100 µl of ice cold distilled water. Samples were
extracted with 1.0 ml extraction buffer (15 g of sodium
tetraphenylboron in 850 ml toluene and 150 ml acetonitrile)
and centrifuged for 2 min in an Eppendorf microcentrifuge,
after which 0.65 ml f the organic phas was c ll cted and

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add d t 5.0 ml scintillati n cocktail (Nati nal Diagn stics, Manville, NJ). Samples w r c unted in a scintillati n count r for 10 min. The amount of pr t in p r culture was determined by a Coomassie pr tein assay (Pierce Rockville, IL).

Northern Blot Analysis

Fibroblasts were seeded onto a 10 cm tissue culture plate and allowed to reach 70-80% confluence. After washing with PBS, total RNA from each culture was isolated by extracting the cells in 0.5 ml of a guanididium isothiocyanate solution as described by Chomczynski and Sachhi, Anal. Biochem. 162:156-159 (1987), incorporated by reference herein. The amount of RNA was quantified by measuring absorbance at 260 nm. Ten to 15 μg of total RNA was loaded onto a 1.2% formaldehyde-agarose gel. Separated RNA was blotted onto nylon membranes (MSI) as described by Sambrook et al., in "Molecular Cloning, A Laboratory Manual", (Ed. Nolan), Cold Spring Harbor Lab. Press, Cold Spring Harbor, NY (1989), incorporated by reference herein. Blots were prehybridized (50% formamide, 5X Denhardt's solution, 5X SSPE, 0.5% SDS, 100 μg/ml denatured herring sperm DNA) for 1-4 hrs at 42°C. Probes were prepared by excising an approximately 2.5 Kb EcoRl fragment from pLChRNL or a 953 bp BamHI fragment from pGCyc (provided by H. Jinnah, University of California, San Diego, CA) to identify dChAt or cyclophilin mRNA, respectively. The fragment was isolated on a 1.0% low-melt agarose gel, purified using Gene Clean (Bio 101, San Diego, CA) and labelled by random priming (Boerhinger Mannheim, Indianapolis, IN) using ^{32}P-dCTP. Approximately 1 x 10^6 cpm/10 ml hybridization buffer was used per blot. The radiolabelled probe was directly applied to the prehybridization solution. Hybridization was performed for 12-20 hrs at 42°C. Afterwards, the blot was washed two times with 6X SSC, 0.1% SDS at ambient room temperature, two times with 1X SSC, 0.1% SDS at 42°C, and finally nce with 0.1X SSC, 0.1% SDS at 65°C. All wash s

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lasted f r 10 min. The washed blot was wrapped in plastic
film and autoradiographed (XAR film, Kodak, Rochester, NY).
The probe was removed for subsequent hybridizations by
incubating the blot in 50% formamide, 10X SSPE at >65°C for 1

5   hr. The blot was rinsed in 2X SSPE before the next
prehybridization. Densimetric scans of autoradiographs were
conducted on a LKB ultrascan XL laser densitometer.

Immunocytochemistry

10

Fibroblasts were seeded at a density of 1 X 10⁴ cells onto
two-welled tissue culture slides (Tissue Tek) and allowed to
grow for two to three days before they were fixed with 4% PBS-
buffered paraformaldehyde. Cultures were permeabilized with

15   0.25% Triton-PBS for 15 min, washed twice with PBS, and
incubated with primary antibody at ambient room temperature
overnight. The primary antibodies used were a rabbit-anti-
dChAT (1:100, from Dr. P. Salvaterra, Duarte, CA), mouse-anti-
vimentin (1:250, Vector Laboratories), or a rabbit-anti-rat

20   ChAT (1:7500, Boehringer Mannheim). In most cases, cultures
were simultaneously incubated with antibodies against vimentin
and one or the other ChAT antibody. After the primary
incubation, cultures were again washed with PBS (twice) and
incubated for 1 hr at 37°C with fluorescent labelled goat-

25   anti-rabbit (1:100, Vector Laboratories) and goat-anti-mouse
(1:100, Chemicon, Temecular, CA), secondary antibodies.
Slides were mounted in Hydromount (National Diagnostics) and
viewed with an Olympus fluorescent microscope.

Serum Starvation and Confluence-Induced Quiescence

30

Rat-1 cells were plated at a density of 2.5 X 10⁵ cells
per 60 mm plate. For serum starvation experiments, cells were
allowed to reach 70-80% confluence before the culture medium

35   was aspirated and replaced with fresh DMEM only. Control
cells were f d normal serum-supplemented medium. Incubation
lasted for 20 hr.

For confluence-induc d quiescence, cells were plat d as described. Cultures were then maintained f r 3, 7, and 14 days after they reach d c nfluence. To feed these cultures, half of the cultur medium was aspirated and replac d with an equal volume of fresh medium. The last feeding was three days before the cells were to be assayed. Cultures containing actively growing cells served as controls. For Northern blot analysis, cells were plated in 10 cm plates and treated as described.

[Methyl]-^{3}H-thymidine incorporation

Incorporation of thymidine was used to monitor the proliferative rate of fibroblasts in logarithmic or quiescent growth state. Cells were plated onto a 10 cm culture dish and incubated with ^{3}[H]-thymidine (25 Ci/mmol; 0.5 μCi/ml; Boehinger Mannheim) for 5 hr. Cultures were subsequently trypsinized, counted using a hemacytometer, pelleted, and resuspended in 1 ml of 0.3 M NaOH. Incubation in the NaOH solution lasted for 30 min. The entire lysate was then pipetted into a scintillation vial containing 10 ml of scintillation fluid and counted on a Mark III liquid scintillation system, model 6881 (Tracor Analytic, Austin, TX).

Measurement of ACh by HPLC

ACh was measured by HPLC with electrochemical detection. ACh was separated from choline on a weak cation exchange column. This column was prepared by loading a reverse phase Chropher Cartridge C18 column (100 mm X 3 mm, Chompack) with laurylsulphate. An enzymatic post-column reactor (10 X 2.1 mm, Bioanalytical Systems, West Lafayette, IN) containing immobilized acetylch linesterase (type VI-S) and choline

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oxidase (Sigma, St. Louis, MO) was us d t convert ACh to
hydrogen peroxid . The hydrogen peroxid was subsequently
measur d by l ctr chemical detection (Bi analytical Systems)
at a platinum electrode held at a potential of +0.5 v against

5 a Ag/AgCl reference electrode. Mobile phase containing 0.1M
potassium phosphate and 0.1 mM tetramethylammonium hydroxide
at pH 8.1 was delivered using a LKB 2248 isocratic pump
(Pharmacia, Piscataway, NJ) at a flow rate of 0.8 ml/min.

10 Medium conditioned by fibroblasts was filtered through a
0.22 μm Millipore filter and injected directly onto the
analytical column without any further sample preparation.
Intracellular ACh levels were measured by resuspending the
pellet, sonicating the suspension, removing cellular debris by

15 centrifugation, and then filtering the sample first through a
0.45 μm and then a 0.22 μm Millipore filter. The remaining
filtrate was then injected onto the analytical column.

Effect of Choline Chloride and Acetyl-dl-Carnitine on ACh
20 Production

Fibroblasts were plated at 1 x 10^5 cells per 35 mm plate
and allowed to reach 70-80% confluency. The medium was
subsequently aspirated and replaced with fresh medium

25 containing the appropriate amount of choline chloride (Sigma)
or acetyl-dl-carnitine (Sigma). Cells were incubated in this
medium for 20 hr. Fibroblasts were harvested and prepared
either for the ChAT assay or for measurement of ACh by HPLC.
Cultures to be analyzed by HPLC had 0.1 mM eserine included in

30 the medium.

Statistical Analysis

Student's t-test and a one way ANOVA statistical analysis
35 (STATView, Palo Alto, CA) were used to determine differences
between experiments. A post-hoc Dunnet t test was used to
d termine individual differences b tween groups.

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Confirmation of dChAT Expression by Rat-1/dChAT Fibroblasts

Rat-1 Fibr blasts were inf cted with retrovirus (Fig. 34) and selected in G418-c ntaining m dium. The presence of dChAT in infected cells was initially confirmed by immunocytochemistry. Uninfected Rat-1 (control) and Rat-1/dChAT fibroblasts were simultaneously stained with antibodies against vimentin and dChAT or vimentin and rat-ChAT (Fig. 35a-f). Vimentin immunoreactivity effectively revealed the morphology of the fibroblasts (Fig. 35a-c). Most Rat-1/dChAT cells were morphologically indistinguishable from controls, displaying a flat, epithelial-like phenotype. Some lines of Rat-1/dChAT fibroblasts were observed to be morphologically distinct from controls, exhibiting an elongated morphology.

As expected, anti-dChAT antibodies labeled only Rat-1/dChAT fibroblasts (compare with Fig. 35d and 35e). Staining of dChAT was evenly distributed throughout the cytoplasm; however, there was an area of more intense immunoreactivity immediately around the nucleus. The nucleus itself was not stained. In addition to staining for the presence of recombinant dChAT, Rat-1 and Rat-1/dChAT cells were also stained for the presence of rat-ChAT. There was no indication of rat-ChAT immunostaining in either control or Rat-1/dChAT cells (Fig. 35f). The lack of rat-ChAT immunoreactivity indicates that there is very little, if any, endogenous ChAT activity in Rat-1 cells. Moreover, these observations demonstrate that the dChAT molecule is immunologically distinct from rat-ChAT. Previous biochemical and molecular studies have reported sequence differences between dChAT and the other mammalian ChATs that have been cloned (Berrard et al., _Proc. Natl. Acad. Sci. USA_ 84:9280-9284 (1987); Itoh et al., _Proc. Natl. Acad. Sci. USA_ 83:4081-4085 (1987)). These data show that dChAT, in addition to catalyzing the formation of ACh, may be used as a
specific marker for dChAT- xpressing fibroblasts that have

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been transplanted into the rat CNS.

T tal RNA from Rat-1 and Rat-1/dChAT fibroblasts was als
compar d by Northern bl t analysis to determine specificity of

5   expression of the transgene. The results from these studies
clearly demonstrate that the mRNA encoding for dChAT is
present only in Rat-1/dChAT fibroblasts (Fig. 36a). The size
of the dChAT mRNA isolated from Rat-1/dChAT cells was
approximately 6 kb. The size of the mRNA is due to the lack

10   of a polyadenylation sequence between the dChAT cistron and
RSV promoter. This allows unimpeded transcription to proceed
from the 5' LTR to the 3' LTR.

Finally, control and Rat-1/dChAT fibroblasts were assayed

15   for ChAT activity. The level of ChAT detected in
Rat-1 cells was widely variable, ranging from no activity to
approximately 6.4 nmoles ACh/hr/mg protein. The average
activity displayed by control cells was approximately 1.9 ±
1.1 nmoles ACh/hr/mg protein (Fig. 36b). In contrast to the

20   low levels of ChAT detected in Rat-1 cells, ChAT activity in
Rat-1/dChAT cells was >1200-fold higher. The average activity
of Rat-1/dChAT cultures was 2397 ± 149 nmoles ACh/hr/mg
protein (Fig. 36b). Moreover, expression of dChAT by
transduced fibroblasts was stable. The activity of ChAT in

25   fibroblasts that were continuously passaged for one year after
infection was similar to that in fibroblasts assayed one week
after infection.

Rat-1/dChAT Cells Produce and Secrete ACh

30

The demonstration that transduced Rat-1 cells express an
active recombinant enzyme capable of acetylating choline in an
in vitro assay does not prove that the cells are capable of
manufacturing and extruding ACh from the cytoplasm.

35   Therefore, the level of ACh found in the culture medium and
within the cells was analyzed by HPLC with electrochemical
d tection. N ACh was f und either within th cells r in the

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nutrient medium f uninfected Rat-1 cells (Fig. 36c). This
result suggests that the ChAT activity detected in Rat-1 cells
is due to a contaminating enzyme which is capable of
acetylating cholin . Carnitine acetyltransferase, an enzyme
5   found in most cells, is capable of transferring the acetyl
moiety from acetyl-CoA to choline (White and Wu, Biochemistry
12:841-846 (1973)). In contrast to the absence of ACh in
control cultures, significant levels of ACh, both intra- and
extracellular, were measured in Rat-1/ChAT cultures (Fig.
10   36c). The average cellular content of ACh was approximately
0.78 $\pm$ 0.23 nmol. Comparatively, approximately 1.7 $\pm$ 0.10
nmol of ACh was found in the culture medium.

These results demonstrate that ACh is actively being
15   manufactured by Rat-1/dChAT fibroblasts and that most of the
ACh is released from the cells into the surrounding culture
medium.

Effect of Choline Chloride and Acetyl-dl-Carnitine on ChAT and
20   ACh Expression

Because choline chloride and acetylcarnitine have been
reported to enhance ACh secretion from neurons or synaptosomes
in various model systems (Gilson and Shimada, Biochem.
25   Pharmacol. 29:167-174 (1980); Imperato, Neurosci. Lett.
107:251-255 (1989); Jenden et al., Science 19:635-637 (1976);
Tucek, J. Neurochem. 44:1-24 (1985)), these drugs were tested
for their ability to modulate ACh secretion and ChAT activity
in Rat-1/dChAT fibroblasts. Choline chloride was used in
30   concentrations ranging from 0.25-500 μM (Fig. 37a). These
concentrations were in addition to that normally found in the
medium (approximately 28 μM) and in the serum (< 2 μM).
Exogenous choline concentrations of 0.25-5.0 μM had no affect
on ACh levels. At 10 μM, both intracellular and secreted ACh
35   concentrations increased (Fig. 37a). Intracellular levels of
choline increased by approximately 2-fold; the concentration
 f ACh f und in th culture medium increased by approximately

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1.8-f ld. Intracellular levels f ACh c ntinued t rise with
increasing concentrations f choline (Fig. 37a). Th addition
 f choline in amounts gr ater than 500 μM did n t cause any
further increase in ACh content.

5

In contrast to the consistent rise in intracellular ACh,
secreted ACh declined back to baseline levels at 50 and 100 μM
choline after increasing by 80% at 10 μM choline (Fig. 37a).
At 200 μM choline, released levels of ACh increased by
10   approximately 2.0-fold. ACh continued to increase at 500 μM
choline, reaching 2.6 times the levels observed in control (no
added choline) cultures. Concentrations above 500 μM choline
did not elicit any further increase in ACh release. The
addition of choline (up to 10 mM) to the medium of uninfected
15   Rat-1 cells did not result in the detection of ACh as
determined by HPLC.

The effect of choline on ChAT activity was also
determined (Fig. 37b). Concentrations of 10, 200 and 500 μM
20   choline were tested. The addition of choline to the culture
medium did not significantly affect the ChAT activity of Rat-
1/dChAT fibroblasts.

The influence of acetyl-dl-carnitine on ACh was also
25   tested. Acetyl-dl-carnitine had no effect on the amount of
ACh produced or released from Rat-1/dChAT fibroblasts or on
ChAT activity.

Effect of Serum Starvation and Confluency on the Expression of
30   dChAT

Most of the characterization of Rat-1/dChAT cells has
been conducted on cultures of proliferating fibroblasts.
However, the ultimate goal is to use fibroblasts for grafting.
35   Fibroblasts that have been transplanted into the brain are
most likely not actively proliferating. Thus, the in vitro
activity of quiescent fibroblasts, which may m re accurat ly

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r flect the conditions found _in_vivo_, should be ass ssed.
This ass ssment provides predictive informati n about the
activity of non-dividing fibroblasts that have been implant d
into the brain. To induce qui sc nce in fibr blast cultures,
Rat-1/dChAT fibroblasts were maintained in either a serum-free
medium or at high density for several days. Although both
methods significantly reduced thymidine incorporation,
maintaining fibroblasts in a post-confluent state for a
prolonged period resulted in much lower thymidine
incorporation (Table 3).

<u>Table 3</u>

[^{3}H]-Thymidine Incorporation

<u>Culture Condition</u>	<u>CPM/10^5 Cells</u>	<u>Cells/ml</u>	<u>% Control</u>
Log, 10% serum (control)	3.6 X 10^4	9.9 X 10^5	---
No Serum	3.7 X 10^{3*}	4.7 X 10^5	10.3
14 DPC, 10% Serum	55.4 ± 8.5*	3.0 X 10^6	<0.2

* Significance of $p < 0.01$
14 DPC = 14 days post confluence

Figure 38a illustrates the effect of serum starvation on
ChAT activity. This treatment did not noticeably affect the
morphology or the health of the fibroblasts. In contrast,
maintaining Rat-1/dChAT fibroblasts in serum-deficient medium
caused a marked reduction in the expression of the transgene.
The absence of serum caused an approximately 80% decrease in
ChAT activity.

Northern blot analysis was conducted to determine if the
steady state level of dChAT mRNA decreased with serum
deprivation. There was a significant decrease (approximately
46%) in the level of dChAT mRNA when serum was withdrawn from
the cultures (Fig. 38b). The same blots were also probed with
a cDNA probe to cyclophilin which served as an int rnal

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contr 1 (McKinnon et al., Mol. Cell. Biol. 7:2148-2154
(1987)). Unexpectedly, th st ady state l vels f cyclophilin
mRNA also app ared t decrease (approximately 43%) in serum-
starved fibroblasts. The absorbance ratio of dChAT t

5   cyclophilin was measured with a densitometer to confirm that
the observed decreases in the steady state levels of dChAT and
cyclophilin mRNA were proportional. Indeed, the ratio of the
relative absorbance of dChAT to cyclophilin was similar in
control and serum-starved cultures (Fig. 38c). The amount of

10   actin and HPRT mRNA was also reduced in serum-starved
fibroblasts.

        Since serum deprivation appeared to affect the general
metabolism of fibroblasts, an alternative method was employed

15   in which quiescence was induced by continuing to maintain
fibroblasts for 3, 7 and 14 days after they reached confluency
(days post confluence, DPC). At 3 DPC, the level of ChAT
declined to 80% of the activity observed in control, growing
cultures (Fig. 39a). The activity of ChAT continued to drop

20   with increasing DPC, decreasing to 50% and 20% of control
levels at 7 and 14 DPC, respectively (Fig. 39a). Maintaining
the cells beyond 14 DPC did not result in a further decline in
ChAT activity.

25   The amount of dChAT mRNA in confluent Rat-1/dChAT
cultures was determined by Northern blot analysis (Fig. 39b).
There was no obvious decrease in the level of dChAT mRNA
isolated from growing (control) or confluent Rat-1 cultures,
however, densitometric analysis revealed that the steady state

30   level of dChAT mRNA decreased by approximately 29% and
approximately 35% at 3 and 14 DPC, respectively.
Interestingly, as the relative levels of dChAT mRNA decreased,
the levels of cyclophilin mRNA increased. As compared to
controls, the relative amount of cyclophilin mRNA isolated

35   from 3 and 14 DPC cultures increased by approximately 84%.
Determination of the dChAT to cyclophilin ratio indicated that
a 60.5% d crease occurred at 3 DPC and a 64% d crease occurr d

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at 14 DPC (Fig. 39c). All numb rs are relativ t the
dChAT/cycl philin rati d termined f r growing cells.

Effect of Choline on the release of ACh from Confluent
5   Fibroblasts

        To assess whether choline could enhance the release of
ACh from confluent fibroblasts, Rat-1/dChAT cells were
maintained for 7 DPC in either control or choline-supplemented
10   (500 μM) medium. Figure 40 illustrates the effects of
maintaining Rat-1/dChAT fibroblasts in a confluent state on
ACh production and release. The overall content of ACh
(intra- and extracellular) in 7 DPC Rat-1/dChAT cultures was
18.9% of that measured in cultures containing mitotic cells.
15   The decrease in total ACh was manifested as a 50.1% and 82.1%
decline in intracellular and released ACh, respectively. In
contrast to confluent cultures maintained in normal medium,
quiescent Rat-1/dChAT fibroblasts maintained in choline-
supplemented medium demonstrated ACh levels that were
20   comparable to the levels of ACh measured in proliferating Rat-
1/dChAT cultures (Fig. 40). Intracellular ACh levels
increased by 138%, as compared to confluent fibroblasts
maintained in normal medium, when exogenous choline was added
to the nutrient medium. Similarly, the amount of
25   extracellular ACh increased by 473%.

        The above results set forth in this example demonstrate
that Rat-1 fibroblasts can be successfully infected with a
retroviral vector containing the cDNA encoding dChAT.
30   Moreover, these cells can manufacture and, more importantly,
release the product of ChAT catalysis, ACh. The production
and secretion of ACh from proliferating fibroblasts can be
modulated if additional choline chloride is added to the
culture medium. These data also show that the expression of
35   the transgene is decreased in quiescent fibroblasts; however,
choline can enhance the release of ACh from these cells.

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Rat-1/dChAT c lls were d v loped so that s me of the
fact rs which may affect ACh pr ducti n could b studied _in_
vitro. Rat-1 fibroblasts w r chosen because they can b
continuously maintained in culture with little, if any, change

5   in their genotype or phenotype. The stability in the
expression of the transgene in these cells is reflected by the
observation that ChAT activity was similar in cells assayed
one week or one year after retroviral infection. A short-term
goal for developing ACh-secreting fibroblasts is to assess the

10 function of these cells following implantation into the CNS.
However, Rat-1 and other fibroblast cell lines implanted into
the rat CNS often form tumors (see Horellou et al., _Neuron_
5:393-402 (1990); Wolff et al., _Proc. Natl. Acad. Sci. USA_
86:9011-9014 (1989)). By contrast, primary dermal fibroblasts

15 can survive for extended periods in the brain (6 mos.) without
any indication of tumor development (see Fisher et al., _Neuron_
6:371-380 (1991); Kajawa et al., _J. Comp. Neurol._ 308:2-13
(1991)). Thus, if ACh-secreting fibroblasts are to be used in
brain transplant paradigms, primary cells should be used.

20 Primary dermal fibroblasts appear to react in a similar manner
to Rat-1/dChAT cells with regards to choline and quiescence
(i.e., increased ACh secretion and decreased ChAT activity,
respectively). Therefore, the Rat-1 fibroblasts used in this
Example serve as an accurate _in vitro_ model for the reaction

25 of primary fibroblasts grown under similar culture conditions.

To enhance the ability of cells transplanted into the CNS
to produce a functional effect, it would be useful if the
secretion of the molecule of interest could be modulated by

30 manipulating the humoral environment within the CNS. In the
in vitro experiments described herein, choline increased the
production and secretion of ACh by Rat-1/dChAT fibroblasts.
Importantly, choline also elicited an increase in ACh
production and release from quiescent (confluent) fibroblasts.

35 This latter observation may provide a means to modulate ACh
production by ChAT-producing cells transplanted into the CNS.
At pres nt, the mechanism behind the incr ase in intra- and

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extracellular ACh caused by exogenous cholin is not known.
H wever, the amount f nzyme, as assessed by m asuring ChAT
activity, is n t affected by incr asing th   xtracellular
concentrati n f choline. These results indicate that the
5   rate of the forward reaction, i.e. towards the formation of
ACh and not the amount of enzyme, is responsible for the
increased level of ACh observed in choline-supplemented
fibroblast cultures.

10   These results suggest that the release of ACh may be
increased in dChAT-expressing fibroblasts that have been
implanted into the brain by administering choline, for example
as a dietary supplement. Choline has been reported to pass
through the blood-brain barrier (Cohen and Wurtman, Life Sci.
15   16:1095-1102 (1975); Cohen and Wurtman, Science 191:561-562
(1976); Wecker and Schmidt, Brain Res. 184:234-238 (1980)).

In addition to the possible modulation of the secretion
of transgenic products, another desirable quality of
20   genetically modified cells is stable, long term expression of
the recombinant gene after the modified cells have been
transplanted. Fibroblasts implanted into the brain surround
themselves with copious amounts of collagen and apparently
cease to proliferate (Kawaja et al., supra, 1991). Current
25   work strongly suggests that the expression of transgenes from
the MoMLV LTR is dramatically decreased in serum starved and
contact-inhibited quiescent fibroblasts. The mechanisms of
action behind serum-starved and confluence-induced quiescence
are not clear, but they do appear to be different. This is
30   evidenced by the observation that dChAT mRNA and ChAT activity
decreases more rapidly in serum starved fibroblasts than in
cultures containing contact inhibited cells.

Current data suggests that once primary fibroblasts are
35   implanted into the brain, the expression from a proviral
transgene in these cells is reduced. This hypothesis is
supported by previ us studies that hav sh wn that TH-

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expressing 208F fibr blasts, which can be immunolabel d for TH
in vitro, are n t effectiv ly stain d nc implanted int the
brain (W lff et al., _supra_, 1989). Primary fibroblasts that
express the gene for GAD also demonstrat a marked decreas in

5   GAD activity when they are transplanted into the brain (Chen
et al., _J. Cell. Biochem._, 45-252-257 (1991)). More recent _in
situ_ hybridization and immunocytochemical studies have
indicated that a residual amount of TH mRNA and protein are
present in late post-transplantation (ten weeks) primary

10   fibroblasts (Fisher et al., _supra_, 1991)). These results,
coupled with those presented in this example, demonstrate that
although the amount of recombinant product decreases in
quiescent fibroblasts, there is a residual amount still
present in the cells. Results show that a baseline level of

15   dChAT activity (approximately 20% of that found in
proliferating cells) exists in quiescent Rat-1/dChAT
fibroblasts. This amount of activity does not decrease by
maintaining the fibroblasts in a confluent state for periods
longer than 14 DPC. Thus, if the activity of the transgene is

20   high enough (as measured in confluent cells _in vitro_), the
amount of activity remaining once the cells have been
transplanted may be enough to elicit a functional effect.
This hypothesis is supported by the observation that increased
amounts of substrate (choline) enhance the release of ACh from

25   quiescent Rat-1/dChAT fibroblasts. Therefore, the drop in the
expression of some transgenes may be compensated for by
defined epigenetic factors.

In conclusion, these results demonstrate that fibroblasts

30   can be genetically altered to produce and secrete ACh. ACh
release can be increased by manipulating the extracellular
concentration of choline, possibly by administering dietary
choline. These data also demonstrate that transgene
expression markedly decreased with the cessation of

35   proliferation _in vitro_.

Example VIII

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Use of Promoters In Quiesc nt Fibroblasts

This example provides a comparison of promoters in growing (Log) and confluent (quiescent) fibroblast cultures.
5   The reporter transgene used in this study was chloramphenicol acetyltransferase (CAT).

Expression Vectors

10   Expression vectors containing the CAT gene under the control of various promoters for determining transgene expression in proliferating and quiescent primary fibroblasts, were obtained. The plasmids, pSV2CAT (Gorman et al., Mol. Cell. Biol. 2:1044-1051 (1982); Subramani and Southern, Anal.
15   Biochem. 135:1 (1983); ATCC No. 37155) in which the CAT gene is under the control of the SV40 promoter plus the SV40 enhancer sequence, and pSV232ACAT (Fig. 41, provided by Dr. D. Jolly and Dr. Theodore Friedmann, University of California, San Diego, CA, constructed from pSV232Agpt described by Fromm
20   and Berg, J. Mol. Appl. Genet. 2:127-135 (1983)), were used. Plasmids pRSVCAT (Rous sarcoma virus long terminal repeat promoter and enhancer, Gorman et al., Proc. Natl. Acad. Sci. USA 79:6777-6781 (1982); ATCC No. 37152), pMLVCAT (Fig. 42, murine Moloney leukemia virus long terminal repeat promoter
25   and enhancer, constructed from pSV232ACAT, pGEM-3 (Promega Corp., Madison, WI) and pN2 (Eglitis et al., Science 230:1345-1398 (1985) and provided by Dr. M. Rosenberg, University of California, San Diego, CA) and pCMVCAT (Fig. 43, human cytomegalovirus immediate early promoter and enhancer,
30   constructed by Dr. M. Rosenberg from pSV232ACAT and pON249 (from E.S. Mocarski, Stanford University School of Medicine, Palo Alto, CA)) were provided by Dr. Friedmann (University of California, San Diego, CA). Collagen promoters were supplied by Dr. de Crombrugghe, M.D. Anderson Cancer Center, Houston,
35   TX. Plasmids pG100 and pAZ1003 (de Crombrugghe and Schmidt, Methods in Enzymology 266:61-76 (1987)), were provided by Dr. d Crombrugghe (Houst n, TX) and contain the CAT gene under

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the c ntrol f mouse α1(I) and α2(I) collag n pr m ters (d
Crombrugghe, _supra_, α2(I); Thompson et al., _Annals New York_
Acad. Sci. 580:454-458 (1990)) α1(I)).

5   Because CAT is a bacterial gene with no mammalian
equivalent, any CAT activity detected in transfected cells is
due to the presence of the transfected plasmid. The level of
CAT enzyme in transfected cells is proportional to the
strength of the promoters (Gorman et al., _Proc. Natl. Acad._
10   _Sci. USA_ 79:6772 (1982)).

Establishment of Fibroblast Cultures

         To obtain rat primary skin fibroblasts, the abdomens of
15   twenty young (200-250g) anesthetized Fischer 344 rats were
soaked with 70% ethanol. 25 X 25 mm biopsies were removed
from the abdomen of each rat and immediately dipped in 70%
alcohol for 2-3 min. The tissue was then transferred to
phosphate-buffered saline (PBS, pH 7.4) to remove the alcohol.
20   The biopsy material was then placed into a solution containing
10 mg/ml of collagenase in PBS and incubated for 15 min. at
37°C. The dermal layer was removed from the epidermal layer
with the aid of fine tweezers and subject to further enzyme
digestion for 0.5 hr. The cells were then pelleted and plated
25   into 35 mm^2 tissue culture dishes and maintained in Dulbecco's
minimal essential medium (DMEM, Gibco, Grand Island, NY)
supplemented with 10% fetal bovine serum (FBS). All cultures
were maintained in a 10% CO_2 atmosphere at 37°C and were
passaged at a split ratio of 1:2 upon reaching > 80% to 90%
30   confluency. Transfected fibroblasts were grown to 70% to 80%
confluency for assay of CAT activity during log phase. For
assay of CAT activity in confluency, cells were grown to 100%
confluency and then maintained for an additional 7 days before
assay.

35

Transfection of Fibroblasts

Fibroblasts w re transfected with th s lected plasmid in
a rati of 10:1 plasmid DNA t pRSVne [R] plasmid using
Lipofectin reagent (BRL, Inc., Gaith rsburg, MD) as d scribed
by F lgn r t al., Proc. Natl. Acad. Sci. USA 84:7413-7417

5   (1987) and Felgner and Holm, Focus 11:21-25 (1988), both
incorporated by reference herein. For each 60 mm dish (5 X 10^5
cells/dish) different amounts of DNA and 30 μg Lipofectin
reagent/3 ml Opti-MEM media (BRL, Inc.) were used.

10   For selection of stable transformants plasmid DNA
containing the neo[R] gene under the control of RSV/LTR was mixed
with vector DNA containing the reporter gene (CAT) under the
control of various promoters at a ratio of 1:10. After
washing once with Opti-MEM, fibroblasts were incubated in the

15   presence of the DNA/Lipofectin mixture for 5 h at 37°C. After
that time, the media were changed with DMEM, 10% FCS and
incubated for 24-36 h before selection.

The stable transformants were selected in the presence of

20   200 μg/ml G418. The transfected cells were passaged when the
plate was at least 50% confluent. Transfected fibroblasts were
split at a 1:2 ratio upon reaching > 80% confluency.

Transfected cells expressing CAT were assayed as

25   described below at both log phase and after confluency.
Results were compared to determine whether the expression of
the CAT transgene remained stable, decreased or increased
after the cells became confluent.

30   Assay for CAT Activity

CAT activity was assayed using a modification of the
method of Sleigh, Anal. Biochem. 162:156-159 (1987),
incorporated by reference herein. The dish containing the

35   transfected fibroblasts was washed in PBS and the cells were
collected by scraping. The cells were resuspended in 0.25M
Tris-HCl, pH 7.8 buff r (100 μl/plate), lysed by sonicati n

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protein f r log and 2494 DPM/μg protein for qui scent cells).
In c ntrast, the levels f CAT m asured in fibr blasts
containing th α2(I) c llagen promoter increased with
quiescence, although the overall expressi n from th se cells

5   was lower. CAT activity in fibroblasts containing the
enhancerless α2(I) collagen promoter (Coll) was approximately
13 DPM/μg and approximately 75 DPM/μg protein for log and
quiescent cells, respectively. This is an increase of
approximately 576%. Fibroblasts containing the α2(I) collagen

10   promoter with the collagen enhancer (Coll(E); described by
Rossi and de Crombrugghe, Proc. Natl. Acad. Sci. (USA)
84:5590-5594 (1981)) exhibited CAT levels of 405 DPM/μg

protein for log and 606 DPM/μg protein for quiescent

15   fibroblasts. This is an increase of approximately 149%.

These results indicate that transgene expression from
viral promoters may decline in quiescent fibroblasts. In
contrast, transgene expression from the α2(I) collagen

20   promoter increases with quiescence. Because fibroblasts
grafted into the brain are nondividing, one way to maximize
stable transgene expression is by using a promoter that is
normally active in quiescent fibroblasts. The α2(I) collagen
promoter is such a promoter. Moreover, the Coll(E) promoter-

25   enhancer is as efficient as the α2(I) promoter in driving
transgene expression under appropriate culture conditions.

These results demonstrate a method for increasing
transgene expression in cells that have ceased dividing when

30   implanted into the body to promote long term stable expression
of therapeutic transgenes.

Example IX
Regulation of Promoters by Cytokines and
35   Anti-inflammatory Agents

Many mon nucl ar phag cyt s and lymphocytes invade

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the CNS after the blood-brain barrier is compromised; for example after the implantation of cells into the brain. Th se cells ar known t s cr te cytokines into the surrounding extrac llular nvironment. The infiltration and accumulation

5   of these cells around grafted material is observed within a week after grafting. Because fibroblasts have been shown to respond to a variety of cytokines this example was performed to assess the role of cytokines in regulating LTR-driven transgene expression in primary rat fibroblasts.

10

This Example describes the use of 1) an anti-inflammatory agent, dexamethasone, to eliminate the effects of cytokines on the steady state levels of LTR-driven proviral mRNA; and 2)

15   the use of the $\alpha2(I)$ collagen (Coll) promoter with the collagen enhancer (Coll(E)) to take advantage of the high levels of cytokines present in the brain after implantation. The transgenes used in this example were Drosophila choline acetyltransferase (dChAT) and chloramphenicol

20   acetyltransferase (CAT).

Methods

The methods used in this Example were as described above

25   for example VIII with the following exceptions:

Lipofection

The method of lipofection was as described above in

30   Example VIII. The plasmids used to transfect fibroblasts in this example were pMLVCAT (LTR promoter supplied by Dr. Friedmann, University of California, San Diego, CA) and pR40 (Coll(E) promoter and enhancer (described in Rossi and de Crombrugghe, Proc. Natl. Acad. Sci. USA 84:5590-5594 (1987),

35   and supplied by Dr. de Crombrugghe, MD Anderson Cancer Center, Houston, TX). The dChAT-fibroblasts were transduced by infecti n with retr viruses as described above f r Exampl

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VII.

Fibroblast cultures

5   Primary skin fibroblast cultures were established from
rat skin as described above in Example VIII with the following
differences. dChAT-fibroblasts were grown to confluence in a
35 mm tissue culture dish using DMEM supplemented with 10% FBS
and 200 µg/ml of G418. Upon reaching confluence, the cells
10   were switched to medium containing 2% serum and maintained for
an additional 7 days. Fibroblasts that were lipofected with
the pMLVCAT or pR40 plasmids were grown to confluence as
described for the dChAT-fibroblasts. However, after reaching
confluence, these cells were maintained in 10% serum for 4
15   days. They were subsequently switched to 2% serum-containing
medium for the final 3 days (for a total of 7 days post-
confluency).

Treatment of Transfected Fibroblasts with Cytokines or Anti-
20   Inflammatory Agent

          Cytokines were added on the 7th day of post-confluency.
TGFβ1 (10 ng/ml), IL-1β (30 µ/ml), and TNFα (150 ng/ml) were
incubated with the cultures for 24 hrs. In addition, the
25   anti-inflammatory agent dexamethasone (25 µM) was added to
fibroblasts alone and in the presence of TGFβ and IL-1β1. The
cells were then taken for Northern blot analysis (dChAT-
fibroblasts) or CAT assay (cells containing the LTR or Coll(E)
promoters). Infγ was incubated as described for the other
30   cytokines; however, for some experiments the medium containing
Infγ was aspirated and replaced with fresh 2% serum-containing
medium. The cells were maintained in this way for an
additional 48 hours before they were processed for Northern
blot or CAT assay. This procedure was used because it was
35   determined that Infγ required this amount of time to function.

          C nflu nt, dChAT- xpressing fibr blasts w re incubat d

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with TGFβ1, TNFα, IL-1β or Infγ as describ d. Total mRNA from
these cultures was isolated and pr bed with a cDNA fragment
that specifically recogniz s proviral mRNA for the Northern
bl t. Fig. 45a demonstrat s that both TGFβ1 (lane 2) and IL-

5   1β (lane 3) significantly decreased the steady state level of
proviral mRNA. TNFα (lane 4), on the other hand, did not
significantly affect the levels of proviral mRNA. Fig. 45b
illustrates the effects of Infγ on dChAT mRNA content. After
24 hrs there was no change in the steady state level of

10   proviral mRNA; however, mRNA levels were markedly reduced 48
hrs after Infγ was removed and replaced with fresh medium.

These results demonstrate that cytokines affect LTR-
driven dChAT expression *in vitro*.

15
Fig. 46 is a Northern blot illustrating that the co-
administration of TGFβ1 and IL-1β to confluent LTR-driven
dChAT-producing fibroblasts significantly decreased the amount
of proviral mRNA contained within the fibroblasts. In

20   contrast, dexamethasone increased the amount of dChAT mRNA
detected by Northern blot analysis. If dChAT-fibroblasts were
simultaneously exposed to dexamethasone, TGFβ1, and IL-1β for
24 hrs, the level of proviral mRNA was similar to that
detected in control cultures. These results suggest that

25   anti-inflammatory agents, including steroids such as
dexamethasone, can mitigate the combined negative effects of
TGFβ1 and IL-1β on the steady state level of proviral mRNA *in
vitro*.

30   As described above, several cytokines downregulate the
expression of transgenes from the LTR. One way to minimize
the effects of these cytokines after grafting fibroblasts into
the brain would be to reduce the inflammatory response using
an anti-inflammatory agent such as dexamethasone. An

35   alternative approach that would not require the administration
of anti-inflammatory drugs is the use of a promoter or
promoters that ar not affected, or are possibly enhanced, by

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the presence of cytokines. One such pr m ter is the α2(I)
collagen promot r. In thes experiments, th CAT gen was
used as th reporter.

5

        Fig. 47 depicts the effects of TGFβ1, TNFα, IL-1β and
Infγ on the expression of CAT in cultures of quiescent
fibroblasts in which the Coll(E) promoter-enhancer was used to
drive transgene expression (Coll(E)-fibroblasts). For these
10   experiments the cultures were maintained in 2% serum
containing medium for the final three days of a seven day
post-confluent culture state. Surprisingly, the activity of
CAT in Coll(E)-fibroblast cultures maintained in 2% serum was
440% greater than comparable cultures maintained in 10% serum-
15   containing medium (Fig. 47a). This activity approached that
measured for LTR (compare with Figs. 44 (Example VIII) and
47a).

        Of the cytokines tested, TGFβ and IL-1β did not appear to
20   significantly affect CAT activity (Fig. 47b). Exposure to
TNFα, on the other hand, resulted in an approximately 32%
increase in CAT activity. In contrast, Coll(E)-fibroblasts
incubated with INFγ displayed an approximately 53% decrease in
CAT activity. This measurement was made 48 hrs after Infγ was
25   removed and replaced with fresh 2% serum-containing medium.

In vivo Expression Using Coll(E)

        LTR-CAT cells (fibroblasts in which the CAT gene is
30   driven by the LTR) and Coll(E)-CAT cells (fibroblasts in which
the CAT gene is driven by the α2(I) collagen promoter-collagen
enhancer) were transplanted into the striatum of adult Fischer
344 rats as described in Example VIII above. Three (3) μl of
LTR-CAT or Coll(E)-CAT cells (200,000 cells/μl; a total of
35   600,000 cells) was injected. LTR-CAT fibroblasts were
injected into the right side, Coll(E)-CAT cells were injected
into th left. At 1 and 4 weeks after implantation, the rats

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wer p rfused, th brains s ction d, and proc ss d for CAT
immunohistochemistry. Th antibody, anti-chloramph nicol, was

used at a dilution of 1:2000 and was purchased from 5 prime-3-
5   prime, Boulder, CO.

As shown in Fig. 48, many strongly stained CAT-positive
fibroblasts were observed in grafts containing either LTR- or
Coll(E)-CAT fibroblasts at 1 week post-transplantation (Fig.
10   48a, Fig. 48b, white arrows). The intensity of staining was
comparable between the two graft types. At 4 weeks, (Fig. 48c
and 48d, white arrows), the staining for CAT was more robust
in Coll(E)-CAT fibroblasts grafts. There were still many
phagocytic cells associated with all grafts at this time point
15   (Arrowheads indicate the graft-host brain interface).

These results suggest that the Coll(E) promoter-enhancer
may provide more stable and stronger expression of transgene
after donor cell implantation into the brain. The presence of
20   phagocytic cells within the center of the grafts at 4 weeks
indicates that cytokines may still be present at high levels.
Thus, one reason for the stronger expression from the Coll(E)
promoter-enhancer as compared to the LTR may be that the
cytokines are acting to increase expression.
25

The data obtained in this example demonstrate that 1)
some cytokines (for example, TGFβ1, IL-1β and Infγ) negatively
affect the level of proviral mRNA, and that 2) dexamethasone
can counteract the negative effect of TGFβ and IL-1β on the
30   steady state level of proviral mRNA and that 3) the Coll(E)
promoter-enhancer may be useful to take advantage of the
levels of cytokines found in the brain after grafting.

The observation that cytokines can downregulate the
35   expression from the LTR suggests that fibroblasts implanted
into the brain may be affected by cytokines that are produced
by bl od-borne mononuclear phagocytes and lymphocytes that

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have penetrated int the brain after grafting. Microglial and endoth lial cells, another source f cyt kines in th brain, may contribute to this scenario. Thus, expressi n f LTR-driven transgenes in grafted fibroblasts may eventually be

5   compromised with time post-transplantation. One way to counteract the detrimental actions of cytokines on the LTR is to use molecules such as anti-inflammatory agents that curtail the production of cytokines. This treatment may not only increase the stability of transgene expression in implanted

10   fibroblasts, but may contribute to the survival of the cells by reducing the immune response.

       An alternative approach takes advantage of cytokines by using a promoter that is up-regulated, or at least not

15   affected, by cytokines. This example demonstrates that TGFβ1 and TNFα do not negatively affect transgene expression (as assessed by CAT activity) in Coll(E)-fibroblasts. In comparison, TNFα increases expression. Infγ had a similar effect on the Coll(E) promoter-enhancer as it did on the LTR

20   (i.e. reduction of transgene expression). Therefore, it may still be necessary to control the release of cytokines such as Infγ from T-lymphocytes, with immunosuppression drugs such as cyclosporin.

25   The data also indicates that expression from the Coll(E) promoter-enhancer is significantly increased in cells maintained in 2% as compared to 10% serum-containing medium. The level of expression is comparable to that observed in fibroblasts containing the LTR. Fibroblasts implanted into

30   the body would be exposed to less serum elements than in culture. Therefore, expression from the Coll(E) promoter-enhancer under certain conditions may be equal to the strongest viral promoters currently used to drive transgene expression.

35

Example X

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Regeneration of Adult Axotomized Neurons

In this Exampl , a new _in vivo_ model is presented to
assess the substrate that supports aberrant axon growth in
5   response to trophic factors in the adult rat CNS. In
addition, the importance of NGF on the regenerative capacities
of rat septal neurons after axotomy is assessed.

This Example demonstrates that adult reactive astrocytes
10   can serve as permissive substrates for axon growth when
supplied by NGF from intrastriatal grafts of genetically
modified primary fibroblasts. In addition, the effects of
grafts of modified primary fibroblasts secreting NGF on the
regenerative capacities of rat septal neurons after axotomy
15   are assessed.

Grafts consisting of primary skin fibroblasts genetically
modified to express and secrete NGF prepared as described
above in Example II were implanted into the striatum of adult
20   rats. Primary fibroblasts were obtained from a skin biopsy of
a female Fischer 344 rat. The cells were maintained under
standard culture conditions and fed DMEM containing 10% fetal
calf serum three times a week. Primary cells were infected
with a murine retroviral vector containing the cDNA for mouse
25   β-NGF, pLN.8RNL, as described above in Example II; infected
cells were selected with the neomyocin analogue G418. Prior
to isolating the cells for grafting, samples of culture media
were taken from control and infected cells. Employing a
sensitive two-site immunoassay (Boerhinger-Mannheim), it was
30   determined that the infected cells produced and secreted 154-
173 pg NGF/hr/10^5 cells; levels of NGF could not be detected in
control media. Female Fischer rats (weighing approximately
175-200 g) were anesthesized with a mixture of ketamine-
xylazine (ketamine, 25 mg/ml; rompun, 1.3 mg/ml; and
35   acepromazine, 0.25 mg/ml). The heads were shaved and placed
in a stereotaxic frame; antiseptic was applied to the head
bef re surgery commenced. Each animal received 3 X 10^5 cells

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in 3 μl of grafting solution (phosphat buffer d saline supplemented with 1 μg/ml of $MgCl_2$ and $CaCl_2$, and 0.1% glucose) into the striatum: NGF-producing cells into the right, and non-infected cells int the left. Aft r survival periods f one, three and eight weeks, the animals were anesthetized and perfused transcardially with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer. Horizontal or sagittal sections through grafts were cut on freezing microtome and processed for NGF receptor immunoreactivity, or were cut on a vibratome and processed for electron microscopic examination.

Genetically modified cells were injected into the right striatum and non-infected control cells into the left striatum. One week after implantation, a plexus of NGF receptor-immunoreactive axons was evident at the caudal pole of striatal grafts composed of NGF-producing fibroblasts, whereas only a small number of enlarged and swollen immunoreactive axonic profiles were observed adjacent to grafts of non-infected control cells (Fig. 49a and 49b). At three weeks, the density of immunoreactive axons surrounding the NGF-producing grafts increased dramatically; a few NGF receptor-immunoreactive profiles completely filled the grafts composed of NGF-producing cells, and the plexus of axonic profiles surrounding the grafts was no longer present (Fig. 49d). These immunoreactive profiles appeared larger than a single axon and usually extended in a vertical fashion. Although grafts of non-infected primary fibroblasts lacked such patterns of immunostained axonic profiles at three and eight weeks after implantation, NGF receptor immunoreactivity was confined to a few blood vessels (Fig. 49c).

Ultrastructural examination revealed that all intrastriatal grafts were composed of primary fibroblasts with extensive rough endoplasmic reticulum and an extracellular matrix filled with collagen (Fig. 50). Capillaries composed of nonfenestrat d nd th lial cells contribut d to the extensive

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vascular plexus within these grafts (Fig. 50). At thr e and
eight weeks after implantation, reactiv astrocytes completely
envel ped the grafts; these astrocytic profiles possess d a
distinct basal lamina on those surfaces apposing the graft

5 environment. This barrier between the CNS neuropil and non-
CNS graft was characteristic of a "glial scar" usually found
following penetrating wounds of the neuropil. Also, many
processes of reactive astrocytes extended into the substance
of the grafts and were often closely associated with

10 capillaries. Within the extracellular matrix of the NGF-
producing grafts only, bundles of unmyelinated axons were
interspersed throughout the grafts; terminals containing
clear, spherical vesicles were occasionally observed as well.
Reactive astrocytic processes laden with filaments were found

15 to envelope these axonic profiles in a convoluted fashion
(Fig. 51a and 51b). Basal lamina surrounded the entire axo-
glial complex and was not evident on opposing plasma membranes
between axons and astrocytes. The incidence of these axo-
glial arrangements was greater at eight weeks than at three

20 weeks following implantation. Axonic profiles were not found
within grafts of non-infected control cells.

These data reveal that NGF receptor-positive axons grow
toward grafts of NGF-producing primary skin fibroblasts.

25 Grafts of non-infected cells, on the other hand, do not elicit
a similar neuronal response. Implants of both types of cells
did, however, induce reactive astrocytes to surround and
encapsulate the grafts before three weeks. Despite the
presence of this glial barrier, unmyelinated axons migrating

30 along elaborate arrangements of reactive astrocytic processes
were found within the NGF-producing grafts only; these axons
were confined exclusively to astrocytic surfaces that lacked a
distinct basal lamina. Although the extracellular matrix of
primary fibroblast implants consists of other conducive

35 substrates for axon growth, e.g. collagen, laminin and
fibronectin, growing axons within the grafts were not
associated with th se components, all of which have been

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previously demonstrat d as conduciv substrates f r axon
growth _in vitro_. Thus, for NGF-sensitiv axons penetrating
grafts f genetically modified primary skin fibr blasts,
reactive astrocytes are the preferred substrate for axon

5   elongation.

These results show that mature reactive astrocytes in the
adult CNS can act as substrates which promote rather than
inhibit axon growth. Employing intrastriatal grafts of

10   genetically modified cells that produce NGF, these data show
that successful axon elongation requires the necessary tropic
and trophic support. Thus, it appears that the availability
of growth-promoting factors, and not the substrates for
growth, is a critical influence during regeneration.

15

To examine the effects of grafts on regeneration of
axons, primary cells genetically modified to express NGF _in_
vivo were established and shown to provide trophic support of
axotomized cholinergic septal neurons. To provide a cellular

20   source of NGF _in vivo_, primary skin fibroblasts genetically
modified to express NGF prepared as described in Example II,
were suspended in a collagen matrix and placed in the cavity
formed by a unilateral ablation of the fimbria-fornix (FF)
pathway, a major route for septal axons projecting to the

25   rostral hippocampus.

Under anesthesia, primary skin fibroblasts were obtained

30   from biopsies of the ventral abdominal wall of a female
Fischer 344 rat and these cells were maintained under standard
culture conditions. Using a retroviral vector containing the
cDNA for mouse β-NGF, pLN.8RNL, primary cells were infected in
culture, and NGF production by these cells was later assessed

35   using a two-site immunoassay (also as described in Example
II). T suspend the cells in a c llag n matrix, cultur s f
NGF-producing and contr l n n-infected primary cells were

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rinsed in phosphat -buffered saline, trypsinized and suspended
in DMEM. The cells were then count d and a total of 10^6 cells
were aliqu t d in medium. Type I c llagen from rat tail
(Sigma) was dissolved with 0.1% acetic acid for a final

5   concentration of 3% collagen. Sodium hydroxide (0.1N) was
added to the cell suspension to make the medium more alkaline,
and the collagen was then added (final concentration of 1%),
mixed and aliquoted into centrifuge tubes. The
collagen/fibroblast matrices were then incubated at 37°C for

10   48 h. Forty female Fischer 344 rats (weighing approximately
175 g) were anesthetized for surgery using a mixture of
ketamine (75 mg/kg), Rompun (4.0 mg/kg) and acepromazine (5.6
mg/kg). Their heads were shaved, and the animals were placed
in a Kopf stereotaxic frame. Surgery was a two-step procedure

15   as described in Example II. First, unilateral aspiration
lesions through the cingulate cortex and the FF pathway were
performed under stereoscopic vision. Then, pieces of the
collagen/fibroblast matrices were cut into small pieces
(approximately 2-3 mm^3) and placed into the wound cavity; other

20   animals received only FF lesions. The animals recovered from
surgery and survived for three, four or eight weeks. After
these survival periods, the animals were deeply anesthetized,
as described above, and were perfused transcardially with 4%
paraformaldehyde in phosphate buffer (0.1% glutaraldehyde was

25   added for ultrastructural cases). The brains were removed,
post-fixed, cut on either a freezing microtome or vibratome,
and the sections (40 to 50 μm in thickness) were processed for
acetylcholinesterase histochemistry, immunohistochemistry and
ultramicrotomy.

30

Only those animals that received a complete unilateral FF
lesion and possessed a graft within the cavity were assessed
for: 1) septal neuronal savings following axotomy; 2)
acetylcholinesterase (AChE) histochemical and NGF receptor

35   immunohistochemical staining in the grafts and hippocampus; 3)
Nissl staining and tyrosine hydroxylase (TH), glial fibrillary
acidic protein (GFAP), and laminin immunohistochemical

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staining in the grafts; 4) retrograde transport of flu rescent
markers from the hippocampus ipsilateral t the lesi n and
grafts of ither NGF-producing r c ntrol n n-infected
fibroblasts; 5) ultrastructural organizati n f the grafts;
5   and 6) ultrastructural distribution of AChE staining in the
deafferented dentate gyrus ipsilateral to grafts of NGF-
producing grafts.

Histochemistry and immunohistochemical detection were
10   performed as follows. Alternate sections through
collagen/fibroblast grafts were stained for Nissl substance
using aqueous 0.5% thionin, stained histochemically for AChE
using a modified method of Hedreen et al., _J. Histochem._
Cytochem. 33:134-140 (1985), and stained immunohistochemically
15   for laminin, glial fibrillary acidic protein (GFAP) and
tyrosine hydroxylase (TH). Sections through the septum were
stained immunohistochemically for NGF receptor, and sections
through the hippocampus were stained histochemically for AChE
and immunohistochemically for NGF receptor and TH.
20

Immunohistochemical detection of NGF receptor, TH and
GFAP used the same protocol. Sections were initially treated
in a solution of 0.6% aqueous hydrogen peroxide for thirty
minutes, briefly rinsed in TBS and incubated in a solution
25   containing TBS, 3% normal horse serum and 0.25% Triton-X for
one hour (this solution was used to dilute all antibodies).
The sections were then incubated for 48 hours at 4°C in a
solution containing one of the following antibodies:
monoclonal 192 IgG to NGF receptor, (Chandler et al., _J. Biol._
30   _Chem._ 259:6882-6889 (1984, 1:100 dilution), monoclonal IgG to
TH (Boehringer-Mannheim; 1:250) and monoclonal IgG to GFAP
(Amersham, 1:100). Control sections were incubated in
solutions lacking primary antisera. All sections were then
rinsed in buffer, incubated in biotinylated horse anti-mouse
35   IgG (1:167) for one hour at room temperature. After another
rinse, the sections were incubated in avidin-biotin complex
(ABC; Vector Laboratories) for on hour. They were then

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rinsed again and reacted in a solution containing 0.025%
diaminobenzidine tetrahydrochloride, 0.5% nick 1 chloride, and
0.18% hydrogen peroxide in TBS for five minutes. The reaction
was stopped by rinsing the sections in buffer.

5

    The sections through the grafts were also stained
immunohistochemically for laminin. Again, the sections were
initially treated with 0.6% aqueous hydrogen peroxide, rinsed,
and incubated in a solution of TBS, 3% normal goat serum and
10 0.25% Triton-X. The sections were then incubated in the same
solution as above with the addition of rabbit anti-human
laminin IgG (1:800 dilution) for 48 hours at 4°C; control
sections were incubated in a solution lacking the primary
antiserum. They were then rinsed and incubated in
15 biotinylated goat anti-rabbit (1:220) for one hour at room
temperature. After another rinse, the sections were incubated
in ABC and reacted as above.

    Three (3) and eight (8) weeks after unilateral ablation
20 of the FF and placement of grafts composed of collagen and
primary fibroblasts, a marked decrease in the population of
NGF receptor-immunoreactive neurons was evident in the
ipsilateral medial septum (Figure 52a and 52b). The lines
drawn through the medial septum (MS) and graft (G) and
25 hippocampal dentate gyrus (DG) represent the level of coronal
sections of photomicrographs in this illustration. Injection
sites for the fluorescent microspheres within the hippocampus
are also shown. A modest savings of NGF receptor-positive
neurons in the ipsilateral (right) septum is evident with NGF-
30 producing grafts (arrowheads indicate septal midline).

    For determination of NGF receptor-immunoreactive septal
neurons saved with grafts of NGF-producing fibroblasts, five
sections, 120 μm apart, through the septal area stained
35 immunohistochemically for NGF receptor were selected for
neuronal cell counting. A counting grid (0.5 X 0.5 mm) was
used t c unt all immunoreactive neur ns in the ipsilateral

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and contralateral medial s ptum. Differences between the
percentage of NGF recept r-p sitive surviving cells per animal
for each group f animals wer assessed by a ne-way ANOVA.
The results are summarized in Table 4, showing percentages of
5 septal neurons saved (contralateral vs. ipsilateral) with
grafts of NGF-producing fibroblasts or control non-infected
fibroblasts in the lesion cavity, or without grafts, three (or
four) and eight weeks following unilateral FF ablation.
Coronal sections through the septum were stained
10 immunohistochemically for NGF receptor to evaluate the
survival of septal neurons. Sections through the fibroblast
grafts were stained immunohistochemically for TH to assess
sympathetic axon ingrowth, for GFAP to assess astrocytic
responses to the grafts, and for laminin to reveal
15 neovascularization. Sections through the grafts and
ipsilateral hippocampus were stained histochemically for AChE
and NGF receptor to examine the regrowth of septal axons.

20

Table 4

| | Post-operative survival periods | |
	3 (or 4) weeks	8 weeks
NGF-producing grafts	68 ± 4*(n=3)	62 ± 7* (n=7)
Control grafts	47 ± 4 (n=4)	47 ± 5 (n=8)
No grafts	44 ± 8 (n=3)	--

30 * Significance at the P < 0.01 level

Table 4 shows that grafts of NGF-producing primary
fibroblasts sustained significantly higher percentages of NGF
receptor-positive septal neurons at three (or four) and eight
35 weeks than did grafts of collagen with control non-infected
primary fibroblasts at the same time periods. The percentages
of NGF rec pt r-immun reactive neurons are comparable with

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control non-inf cted grafts and without grafts. Thus, while
the degree of savings with NGF-producing cells was modest,
these results provide indirect evid nce for the _in vivo_
expression of the transgene for NGF by genetically modified

5   primary fibroblasts. This expression should enable the cells
to provide trophic support to axotomized cholinergic septal
neurons.

Regeneration of septal axons within the grafts and

10   deafferented hippocampal dentate gyrus was assessed through
the detection of AChE and NGF receptor staining. Grafts of
NGF-producing fibroblasts usually stained moderately to
intensely for AChE (Fig. 52c). Grafts of control, non-
infected primary fibroblasts, on the other hand, often lacked

15   AChE reactivity (Fig. 52d). Note that the NGF-producing graft
possesses a densely stained perimeter, whereas the control
graft lacks AChE reactivity (dotted line represents
approximate boundary of the graft).

20   Three weeks after the transection of the FF unilaterally
and placement of control, non-infected fibroblasts/collagen
grafts within the cavity, the deafferented dentate gyrus was
devoid of axons stained for AChE and NGF receptor. This was
in stark contrast to the prominent staining for AChE (Fig.

25   52e) and NGF receptor seen in the normal dentate gyrus. The
normal pattern of AChE staining reveals a robust input to all
layers of the dentate gyrus. By eight weeks after
lesion/implantation, the deafferented dentate gyrus still
lacked AChE-positive axons (Fig. 52g) but now possessed large-

30   diameter axons stained immunohistochemically for NGF receptor,
particularly in the polymorphic layer of the dentate gyrus;
these axons are reminiscent of sympathetic axons that sprout
into the hippocampus following FF lesions (see Batchelor et
al., _J. Comp. Neurol._ 284:187 (1989)). In two cases, AChE-

35   positive axons were observed traversing beneath the control
grafts from the contralateral to the deafferented hippocampus.
Three weeks after the unilateral transection of the FF and

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placement of NGF-producing fibr blasts in collagen, a moderate plexus f AChE-positive axons was evident in the medial and rostral asp cts f th deafferent d dentate gyrus. By eight weeks, the density of AChE-positive fibers increased markedly,

5 yet most axons were still confined to the rostromedial dentate gyrus. In certain cases, however, a robust plexus of topographically organized AChE-positive axons was observed within the dentate gyrus and CA2-3 fields (Fig. 52f). Although the pattern of reinnervation was comparable to the

10 distribution of AChE-positive septal axons in the normal undamaged hippocampus (Fig. 52e), the density of axons was usually less. In those cases with NGF-producing grafts, large-diameter axons stained for NGF receptor were also observed in the polymorphic layer of the deafferented dentate

15 gyrus, usually in the caudal portions of the hippocampus.

Although the pattern of AChE staining within the NGF-producing and control grafts was strikingly different, both

20 types of grafts possessed similar cellular populations and immunostaining for TH, GFAP and laminin. Nissl staining showed that all grafts were composed of dense bundles of collagen with a variety of different cell types within and surrounding the grafts, including fibroblasts, lymphocytes,

25 mast cells, plasma cells, astrocytes and endothelial cells of capillaries. Also, both types of grafts possessed a small number of TH-immunoreactive axons, particularly within the dense collagen bundles. Astrocytic processes stained immunohistochemically for GFAP were seen extending from the

30 damaged neural tissue around the wound cavity and penetrating into the outer aspects of the grafts; occasionally, GFAP-positive cells were seen within the grafts as well. Laminin immunostaining of grafts of NGF-producing and non-infected primary fibroblasts revealed an extensive host-derived

35 vascular network. Also, elongated laminin-immunoreactive cell bodies, perhaps Schwann cells, were f und within and ar und th dens collagen bundl s of both types f grafts.

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To determine the origin of those septal neurons that
regenerate new axons following FF ablation and reinnervate th
deafferented hippocampus, stereotaxic plac ments of
fluorescent microspheres were made into the ipsilateral
5   dentate gyrus and CA2-3 fields. Nine (9) deeply anesthetized
animals with grafts of either NGF-producing (n=5) or control,
non-infected (n=4) fibroblasts received two stereotaxic
placements of rhodamine-fluorescent microspheres (200 nl/site)
into the dentate gyrus and CA2-3 fields in the hippocampus
10   ipsilateral to the lesion and graft eight weeks after surgery.
Following a 48h recovery, the animals were again anesthetized
and perfused transcardially with 4% paraformaldehyde. The
brains were sectioned coronally on a freezing microtome, and
retrogradely labeled neurons within the septal areas were
15   viewed with a fluorescence microscope. The schematic
illustration in Fig. 52 shows the injection sites.

Most retrogradely fluorescently labeled elongated neurons
were found within the ipsilateral medial septum and diagonal
20   band areas, while a smaller number were observed in the septal
midline and in the contralateral septal nuclei (Fig. 53).
Cases with grafts of control, non-infected fibroblasts did not
possess retrogradely labeled neurons in any area of septal
region.
25

At the ultrastructural level, the grafts consisted of
dense collagen bundles with a loose outer reticular
arrangement eight weeks after implantation. Fibroblasts were
observed both within the dense and reticular collagenous
30   formations, and attenuated processes of these cells enveloped
dense accumulations of collagen in both areas. Capillaries
composed of non-fenestrated endothelial cells surrounded by
basal lamina were found throughout the grafts. Several types
of cells were also observed within the grafts, including
35   lymphocytes, plasma cells, mast cells, and phagocytes.
Astrocytes and their process s were found pr dominantly within
the lo se reticulum around the dense collagen center. Th

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most prominent difference b twe n grafts f NGF-pr ducing and non-infected fibr blasts was the number f unmyelinat d axons, i.e. within an area of 960 μm^2, 1625 axons were found in th NGF-producing grafts compared with 329 axons in the control

5   grafts. Ultrathin sections of either NGF-producing (n=3 different cases) or control, non-infected (n=3 different cases) fibroblast/collagen grafts were examined and the total number of axons were counted in 10 grid squares (2 parallel columns of 5 grid squares, a total area of 960 μm^2, with graft

10   tissue of the dense and loose collagen areas). Among the three cases of NGF-producing grafts, 1625 axons were found in

comparison to 329 axons among the three cases of control, non-infected grafts.

15

       As shown in Figs. 54a-Fig. 54c large numbers of axons were surrounded by or passed along narrow processes of astrocytes (*) that contain intermediate filaments. These astrocytic profiles possess basal lamina (arrowheads) on those

20   cell surfaces facing the graft extracellular matrix. The axons are usually found apposing those astrocytic surfaces lacking a basal lamina. Such axons were also found near the cell bodies of the astrocytes and other cells, including fibroblasts (F) (Fig. 54d) and other astrocyte-like cells and

25   their processes. Still other unmyelinated axons were closely associated with the basal lamina of the endothelial cells of capillaries (C) or found within the extracellular matrix of the NGF-producing grafts; collagen fibrils are distributed among the axons (Fig. 54e). Within grafts of either NGF-

30   producing or control fibroblasts, small numbers of unmyelinated axons enveloped by Schwann cells (S) and their processes were observed within the reticular areas (L = lumen of capillary) (Fig. 54f). Axons observed within the dense collagen area of both types of grafts were usually ensheathed

35   within attenuated glial profiles. Occasionally, control grafts als possessed a very small populati n of axons within the extracellular matrix.

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Ultrastructural examination of the deafferented
hippocampus following the placement of NGF-pr ducing grafts
revealed a spars distribution of AChE reactivity within the
dentate neuropil. The electron-dense reaction production was
5   localized to the plasma membranes of small-diameter,
unmyelinated axons and terminal containing clear spherical
vesicles; these stained axonic profiles were found
predominantly in the molecular layers of the dentate gyrus
(Fig. 55). Dispersed throughout the dentate neuropil were
10   clusters of AChE-positive unmyelinated axons, and these axonal
aggregates were usually found near astrocytic processes.
Occasionally, axon terminals possessing reactivity for AChE
formed synaptic contacts with dendritic shafts and spines.
The symmetry of contacts between AChE-positive terminals and
15   dendritic profiles of granular neurons, however, was usually
obscured by the localization of reaction product at the site
of apposition. Since sections of the deafferented dentate
gyrus of those animals with control non-infected cells lacked
AChE reactivity, further analysis was not conducted at the
20   ultrastructural level.

In particular, Fig. 55 shows a coronal section (40 μm) of
the dentate gyrus stained for AChE, taken immediately adjacent
to that tissue examined at the ultrastructural level. AChE-
25   positive fibers are evident in the inner (IM) and outer (OM)
molecular layers and the granular layer (G). AChE-positive
somata and fibers are also found in the polymorphic layer (P).
Fig. 55b and 55c show that clusters of AChE-positive
unmyelinated axons are found throughout the granular (B) and
30   molecular (C) layers. Fig. 55d, 55e and 55f show that AChE-
positive terminals form synaptic contacts (arrowheads) with
dendritic shafts (Sh) and spines (Sp) in the molecular layers.
Astrocytic processes (As) are usually observed near clusters
of AChE-positive axons and terminals in the dentate gyrus.
35

These results demonstrate the importance of
trophic/tropic substances such as NGF in th regenerative

capacity f septal cholin rgic neurons, as assessed by the
percentag of NGF recept r-positive neur ns saved within the
medial septum and the reinnervation of the hippocampal dentate
gyrus following a unilateral FF lesion and placement of grafts

5   composed of a collagen matrix with NGF-producing primary
fibroblasts. Intracerebral grafts of NGF-producing primary
fibroblasts are thus capable of preventing the retrograde
degeneration of cholinergic septal neurons after axotomy like
grafts of NGF-producing immortalized fibroblasts as shown in

10 Example II herein, and infusions of exogenous NGF into the
lateral ventricle (Hefti, _J. Neurosci._ 6:2155 (1986); Kromer,
Science 235:214 (1987) and Williams et al., _Proc. Natl. Acad._
Sci. USA 83:9231 (1986)). The percentages of cells saved with
NGF-producing primary fibroblasts in a collagen matrix,

15 however, is much lower than that achieved with
intraventricular infusions of NGF or implants of NGF-producing
immortalized fibroblasts. This may be due to 1) a small
number of cells actually implanted within the collagen matrix
resulting in a lower level of NGF production; and 2) a

20 possible down-regulation of the transgene following
implantation.

These results show that, like other different types of
tissues which support new axon growth from septal neurons,

25 grafts of NGF-producing fibroblasts within a collagen matrix
also support the growth of septal fibers, such that a sparse
reinnervation of the hippocampus is evident at three weeks.
By eight weeks, however, the density of axons within the
hippocampus is clearly greater than that seen at three weeks.

30 Implants of control cells in collagen offer little or no
support, since the ingrowth of septal AChE-positive fibers to
the hippocampus is negligible and no labeled cells are found
in the septal area ipsilateral to the dentate gyrus following
placements of retrograde tracer. Likewise, grafts of

35 acellular peripheral nerve (i.e., those lacking Schwann cells)
cannot support the gr wth of septal axons (Hagg et al., _Exp._
Neurol. 112:79 (1991)). Together, these data reveal that

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septal ax ns are able to use many different graft
environments, consisting of both cellular and extrac llular
substrates for growth.

5

     These data also show that collagen grafts containing
primary skin fibroblasts induce a similar ingrowth of
capillaries with basal lamina, astrocytes and Schwann cells,
regardless of whether the primary cells are genetically
10   modified to produce NGF or not. Moreover, the extracellular
matrices of both types of grafts are similar, such that the
patterns of laminin immunostaining and collagen distribution
are comparable. The real differences between NGF-producing
grafts and control grafts lie in the robust staining for AChE
15   and abundance of unmyelinated axons within the NGF-producing
grafts. First, prominent AChE reactivity is only evident in
NGF-producing grafts; control grafts usually lack AChE
staining. Both types of grafts, however, possess a sparse
population of TH-immunoreactive fibers, as well as axons
20   enveloped within Schwann cells and their processes; most axons
associated with these glial elements may represent TH-positive
sympathetic ingrowth. Second, NGF-producing grafts possess
numerous axons that are ensheathed by astrocytic processes,
pass along the basal lamina of capillaries and astrocytes, or
25   extend within the loose arrangements of collagen. Control
grafts, on the other hand, only possess a very small
population of axons within the extracellular environment.
From these collective data, it appears that NGF-sensitive
axons arising from perturbed septal neurons require the
30   availability of NGF and a permissive graft environment for new
growth. Using grafts of genetically modified cells that
produce NGF in vivo regenerating septal axons have been shown
herein to grow on a variety of different substrates only in
its presence. Without elevated levels of NGF, axons do not
35   regenerate in response to grafts consisting of collagen and
control n n-inf cted fibr blasts, even though the same
cellular and extracellular substrates are available.

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Previous studies examining the regenerative capacity f
septal cholinergic neurons in the rat following axotomy and
grafting have referred to the "reinnervation" f the
deafferented hippocampus with certain bridging environments
5   (Hagg et al., Exp. Neurol. 112:79 (1991)). To date, however,
no investigation has provided direct morphological evidence
that AChE-positive septal axons within the hippocampus
actually innervate postsynaptic targets. Ultrastructural data
presented herein reveal that axons stained for AChE activity
10   are sparsely distributed within the deafferented dentate gyrus
eight weeks after FF lesion and grafting of NGF-producing
fibroblasts. These axons are often seen in clusters of two or
more. The ultrastructural organization of AChE-positive
septal axons is similar to recent observations that septal
15   axons stained immunohistochemically for NGF receptor also form
small aggregates within the normal rat dentate gyrus (Kawaja
and Gage, J. Comp. Neurol. 307:517 (1991)). In addition,
these newly-formed AChE-positive axons give rise to terminals
that form synaptic contacts with dendritic shafts and spines
20   in the deafferented dentate gyrus. The pattern of synaptic
contacts between septal cholinergic axons and granular
dendritic profiles is comparable to that normally found in the
rat dentate gyrus; axosomatic contacts between cholinergic
axons and granular neurons are rare (Shute and Lewis,
25   Zellforsch. Mikrosk. 69:334 (1966); and Clarke, Brain Res.
360:349 (1985)). It is worth noting that when grafts of fetal
septum are implanted within the adult rat dentate gyrus after
FF ablation, cholinergic axons from the grafts innervate the
somata of granular neurons at higher frequency than normal
30   (Clarke et al., Brain Res. 369:151 (1986)). Further, the
number of graft-derived cholinergic axons contacting dendritic
profiles decreases dramatically (Id). From these data, it
appears that while the cholinergic axons from fetal septal
grafts within the deafferented dentate gyrus form unique
35   synaptic arrangements with granular neurons, septal axons that
regenerate acr ss NGF-rich grafts f collagen and fibroblasts
recapitulat a normal synaptic organization within the dentate

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gyrus (i.e., AChE-positive septal axons terminate
predominantly on dendritic shafts and spines).

5   The results presented in this example demonstrate that
grafts of NGF-producing fibroblasts in a collagen matrix
sustain damaged cholinergic neurons of the rat medial septum,
provide a conducive environment for regrowing NGF-sensitive
axons arising from these neurons and influence the
reinnervation of the deafferented granular neurons of the
10   dentate gyrus by septal axons. In particular, these grafts
induce axonal elongation and synaptic connectivity.
Regenerating NGF-sensitive septal axons use both cellular and
matrix substrates for growth within grafts consisting of NGF-
producing primary fibroblasts and collagen. Without elevated
15   levels of NGF (i.e. using control fibroblast/collagen grafts),
a higher proportion of septal neurons undergo degeneration and
axons fail to grow, despite the presence of conducive
substrates.

20   In addition to NGF, other neurotrophic factors, such as
brain-derived growth factor (BDNF), neurotrophin (NT)-3, NT-4,
and ciliary neuronal trophic factor (CNTF), may be used to
sustain axotomized neurons and promote axon regrowth of other
neuronal populations in the CNS.
25

It is apparent that many modifications and variations of
this invention as set forth above may be made without
departing from the spirit and scope of the present invention.
The specific embodiments described are given by way of example
30   only and the invention is limited only by the terms of the
appended claims.

We claim:

1. A method for treating defective, diseased or damaged
5   cells in the mammalian central nervous system comprising
grafting donor cells into the central nervous system, said
donor cells genetically modified to produce functional
molecules in a sufficient amount to ameliorate said defective,
diseased or damaged cells in the central nervous system.

10

2.   The method of claim 1 wherein the step of grafting said
donor cells comprises introducing said donor cells into the
brain of a subject.

15   3.   The method of claim 1 wherein the step of grafting said
donor cells comprises introducing said donor cells into the
spinal cord of a subject.

4.   The method of claim 2 wherein said introducing comprises
20   intracerebral, intraventricular, subdural space, putamen,
nucleus basalis, hippocampus, cortex, striatal, caudate and
intravenous introduction.

5.   The method of claim 1 wherein said donor cells are
25   modified by insertion of at least one therapeutic transgene
into said cells.

6.   The method of claim 5 wherein said step of insertion
comprises inserting a vector carrying said transgene, said
30   vector selected from the group consisting of viral,
retroviral, and neurotropic virus.

7.   The method of claim 6 wherein said vector is a herpes
virus vector.

35

8.   The method of claim 6 wherein said vctor is a rabies

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virus vector.

9.   The method of claim 6 wherein said vector is a viral
vector.

5

10.   The method of claim 6 wherein said vector is a
retroviral vector.

11.   The method of claim 10 wherein said retroviral vector is
10   the retroviral vector pLN.8RNL having a final construction as
shown in Figure 12.

12.   The method of claim 10 wherein said retroviral vector is
the retroviral vector pLThRNL having a final construction as
15   shown in Figure 16.

13.   The method of claim 5 wherein said step of insertion into
donor cells comprises nonviral physical transfection of DNA
encoding a transgene.

20

14.   The method of claim 13 wherein said nonviral physical
transfection comprises microinjection of DNA encoding a
transgene.

25   15.   The method of claim 5 wherein said step of insertion into
donor cells comprises electroporation.

16.   The method of claim 5 wherein said step of insertion into
donor cells comprises chemically mediated transfection.

30

17.   The method of claim 16 wherein said chemically mediated
transfection comprises calcium phosphate transfection.

35   18.   The method of claim 5 wherein said step of insertion into
donor cells c mprises liposomal mediated transfection.

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19.   The method of claim 5 wherein said step of insertion into donor cells comprises lipofection.

20.   The method of claim 1 wherein said molecules are selected
5   from the group consisting of growth factors, enzymes, gangliosides, antibiotics, neurotransmitters, neurohormones, toxins, neurite promoting molecules, antimetabolites and precursors of said molecules.

10   21.   The method of claim 5 wherein said therapeutic transgenes are selected from the group consisting of tyrosine hydroxylase, NGF, tryptophan hydroxylase, ChAT, GABA-decarboxylase, enkephlin, Dopa decarboxylase (AADC), ciliary neuronal trophic factor (CNTF), brain derived neurotrophic
15   factor (BDNF), neurotrophin (NT)-3, NT-4, and basic fibroblast growth factor (bFGF).

22.   The method of claim 1 further comprising co-administration of a therapeutic agent for treating said
20   disease or damage to the central nervous system.

23.   The method of claim 22 wherein said therapeutic agent is selected from the group consisting of growth factors, gangliosides, antibiotics, neurotransmitters, neurohormones,
25   toxins, antimetabolites, neurite promoting molecules and precursors of these agents.

24.   The method of claim 22 wherein said therapeutic agent is cellular matter.
30

25.   The method of claim 24 wherein said cellular matter is selected from the group consisting of adrenal chromaffin
35   cells, fetal brain tissue cells and placental cells.

26.   The method f claim 1 further comprising implanting

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material to the site of said damage or disease, said material
to facilitate reconnection or ameliorative interactions f
injur d neurons.

5   27. The method of claim 26 wherein said material is selected
from the group consisting of homogenate of brain, homogenate
of placenta, collagen, whole cells, synthetic material,
neurite promoting extracellular matrix, and genetically
modified donor cells.

10

28. The method of claim 1 wherein said donor cells are
selected from the group consisting of fibroblasts, neurons,
glial cells, keratinocytes, hepatocytes, ependymal cells, bone
marrow cells, hippocampal cells, olfactory mucosa cells, stem
15   cells, adrenal cells, connective cells, leukocytes and
chromaffin cells.

29. The method of claim 1 wherein said donor cells comprise a
mixture of cell types from different anatomical regions.

20

30. The method of claim 1 wherein said cells are primary
cells.

31. The method of claim 1 wherein said cells are immortalized
25   cells.

32. The method of claim 1 wherein the step of grafting said
donor cells comprises grafting from approximately 10^4 to
approximately 10^8 cells per graft.

30

33. The method of claim 1 wherein said donor cells consist of
cells that have been passaged from approximately 2 to
approximately 20 times.

35   34. The method of claim 1 wherein the step of grafting
comprises multiple grafting of genetically modified donor
cells in sev ral different sites.

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35.　The m thod of claim 34 wherein said d nor cells comprise a mixture f transgenes ins rted into said cells.

5　　36.　The method of claim 34 wherein said donor cells comprise a mixture of cell types.

37.　The method of claim 1 wherein the step of grafting comprises multiple grafting of genetically modified donor
10　　cells into a single site.

38.　The method of claim 37 wherein said donor cells comprise a mixture of cell types.

15　　39.　The method of claim 6 wherein said vector carries a promoter to enhance expression of said transgene when said donor cells are quiescent.

40.　The method of claim 39 wherein said promoter is a
20　　collagen promoter.

41.　The method of claim 40 wherein said collagen promoter is selected from the group consisting of $\alpha1(I)$ and $\alpha2(I)$.

25　　42.　The method of claim 39 wherein said vector further carries an enhancer sequence to increase the activity of said promoter.

43.　The method of claim 42 wherein said enhancer sequence is
30　　SV40 enhancer sequence.

44.　The method of claim 42 wherein said enhancer sequence is a collagen promoter enhancer sequence.

35　　45.　The method of claim 44 wherein said enhancer sequence is a $\alpha2(I)$ collagen enhancer sequence.

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46.   The method of claim 39 further c mprising the
administrati n of cyt kines to regulate the expr ssi n of said
molecules.

5   47.   The method of claim 46 wherein said cytokines are
selected from the group consisting of interleukin-1β,
interferon-α, tumor necrosis factor-α, tumor growth factor-β,
basic fibroblast growth factor (bFGF) and epidermal growth
factor (EGF).

10

48.   The method of claim 1 further comprising the use of
growth factors to maintain survival of the donor cells in the
mammalian CNS.

15   49.   The method of claim 48 wherein said growth factors are
bFGF or EGF.

50.   The method of claim 1 further comprising regulation of
the secretion of said functional molecules by administration
20   of a precursor for said molecules.

51.   The method of claim 50 wherein said functional molecules
are acetylcholine, and said precursor is choline.

25   52.   The method of claim 1 wherein said donor cells are
primary fibroblasts.

53.   The method of claim 52 wherein said donor cells are
primate fibroblasts.

30

54.   The method of claim 52 wherein said donor cells are human
fibroblasts.

55.   A method for treating defective, diseased or damaged
35   cells in the mammalian central nervous system comprising:

       a) grafting fibr blasts genetically m dified with a

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v ct r containing ChAT cDNA to express acetylch line into the
central nervous system of a mammal having defective, diseased
or damaged cells in the central nervous system; and

5   b) administering choline chloride orally to maintain and
enhance secretion of said acetylcholine from said fibroblasts.

56.   The method of claim 55 wherein said method is used to
treat a patient suffering from Alzheimer's disease.

10

57.   A method for enhancing the expression of a therapeutic
transgene product from quiescent donor cells grafted into a
mammalian central nervous system, said donor cells genetically
modified by insertion of a therapeutic transgene via a vector
15   carrying said transgene, comprising inserting a quiescent
promoter in said vector.

58.   The method of claim 57 wherein said promoter is a non-
viral promoter.

20

59.   The method of claim 58 wherein said promoter is a
collagen promoter.

25

60.   The method of claim 59 wherein said promoter is selected
from the group consisting of α1(I) collagen and α2(I) collagen
promoters.

30   61.   The method of claim 57 wherein the expression of said
therapeutic transgene is further enhanced using an anti-
inflammatory agent to reduce the production of cytokines.

62.   The method of claim 61 wherein said anti-inflammatory
35   agent is a steroid.

63.   Th method of claim 62 wherein said anti-inflammat ry

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agent is dexamethasone.

64.   The method of claim 57 further comprising the use of an immunosuppression agent to reduce the production of cytokines.

5

65.   The method of claim 64 wherein said immunosuppression agent is cyclosporin.

66. The method of claim 64 wherein said cytokines are Infγ.

10

67.   A method for treating defective, diseased or damaged cells in the mammalian central nervous system comprising grafting genetically modified primary fibroblasts into the central nervous system, said fibroblasts modified by insertion

15   of a therapeutic transgene carried in a retroviral vector to produce the product of said transgene in a sufficient amount to ameliorate said defective, diseased or damaged cells.

68.   The method of claim 67 wherein said vector is the

20   retroviral vector pLN.8RNL having a final construction as shown in Figure 12.

69.   The method of claim 67 wherein said vector is the

25   retroviral vector pLThRNL having a final construction as shown in Figure 16.

70.   The method of claim 67 wherein said transgene is tyrosine hydroxylase.

30

71.   The method of claim 1 wherein said donor cells are cells from the same species of mammal receiving the cells.

72.   The method of claim 1 wherein said donor cells are cells

35   from a different species of mammal receiving the cells.

73.   The meth d of claim 72 wherein immunological reactions to

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said don r cells are suppressed.

74.   The method f claim 73 wherein an immunosuppression agent
is administered.

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FIG. 1

ESTABLISHED
CELL LINE

GENE EXPRESSION
AND MOLECULAR
CHARACTERIZATION

PRIMARY
CELL

VECTOR

IN VITRO
SELECTION

INFECTED CELLS
AND UNINFECTED
CONTROL CELLS

ENGRAFTING OF
GENETICALLY
MODIFIED CELLS

INTRACEREBRAL
GRAFTING

DISSECTION
OF GRAFT

HISTOLOGICAL
EVALUATION

RE-CULTURE
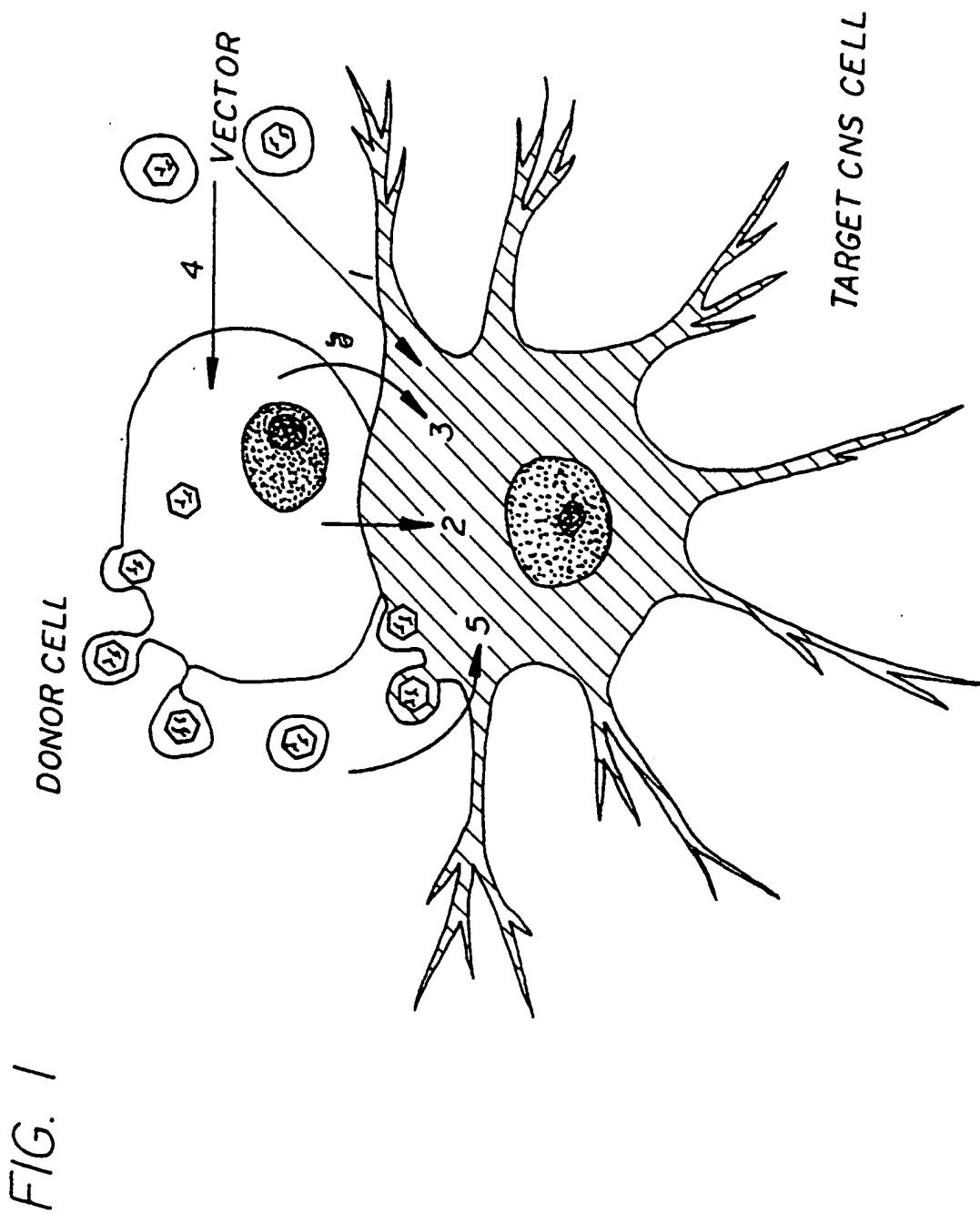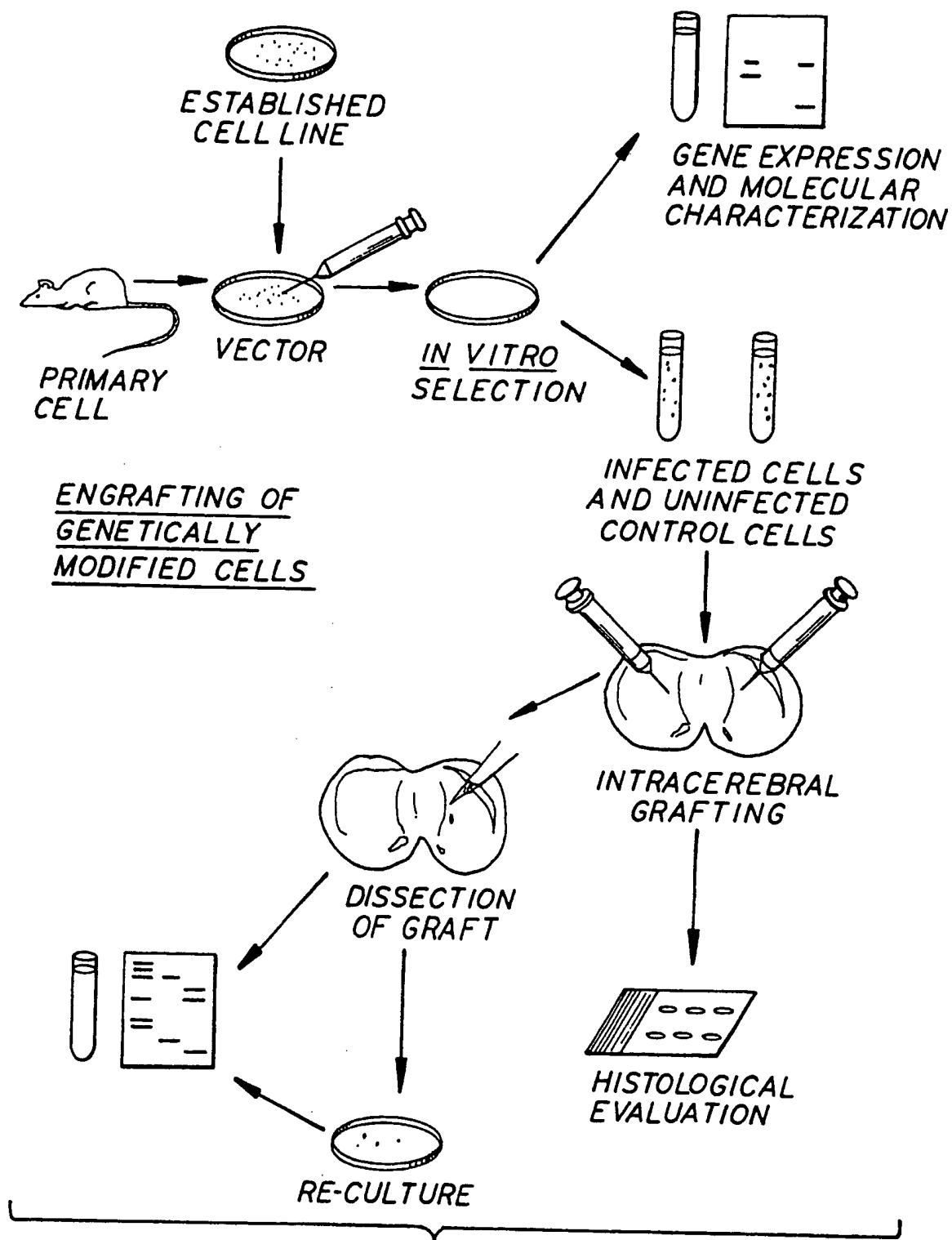
FIG. 2

FIG. 3
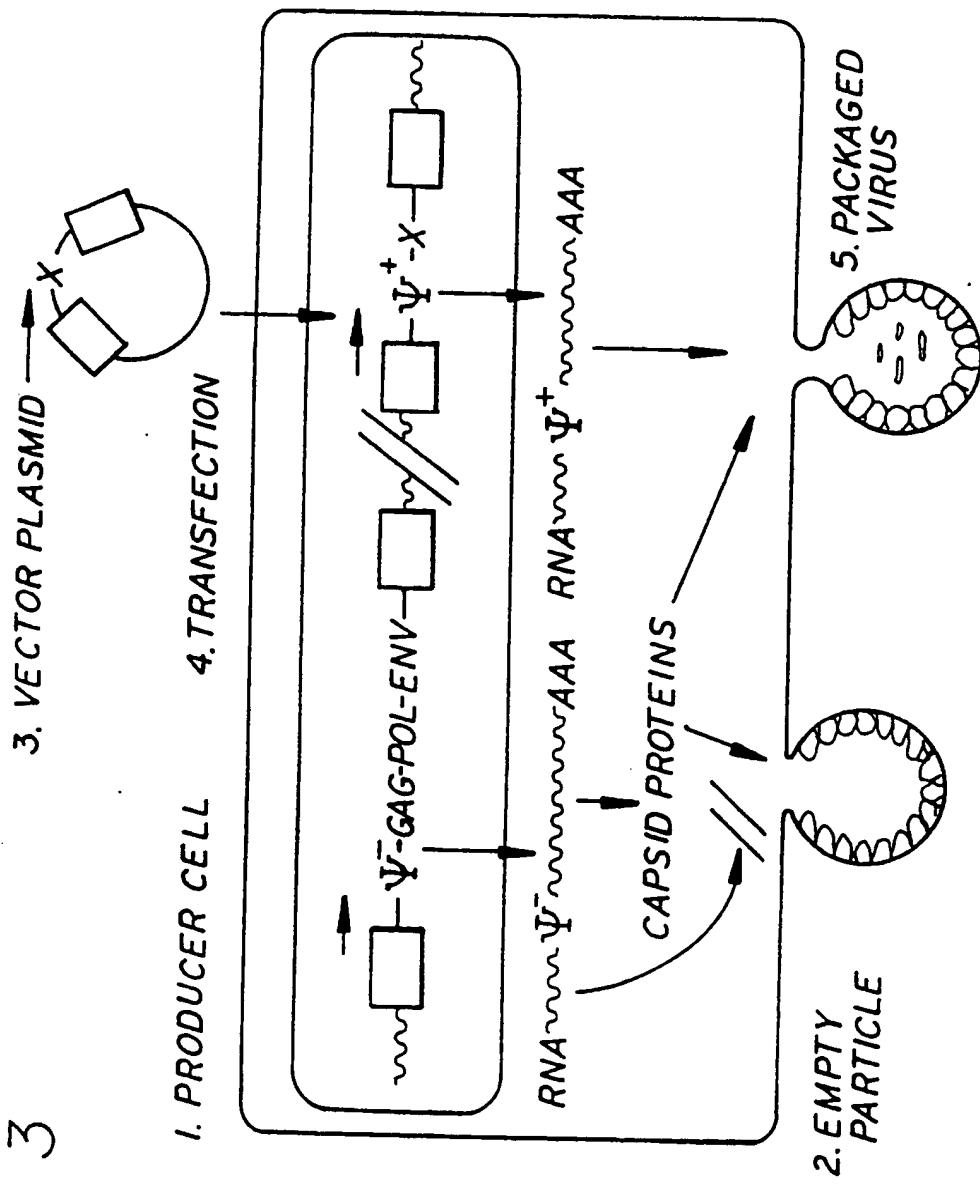
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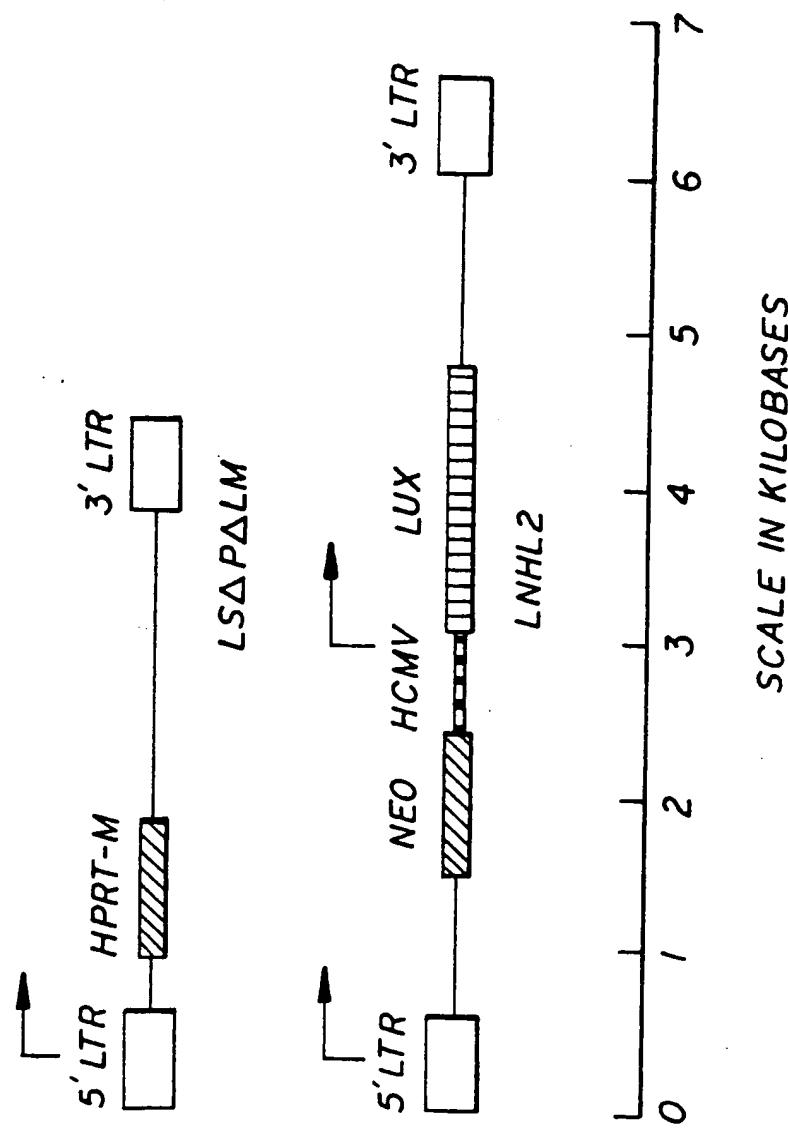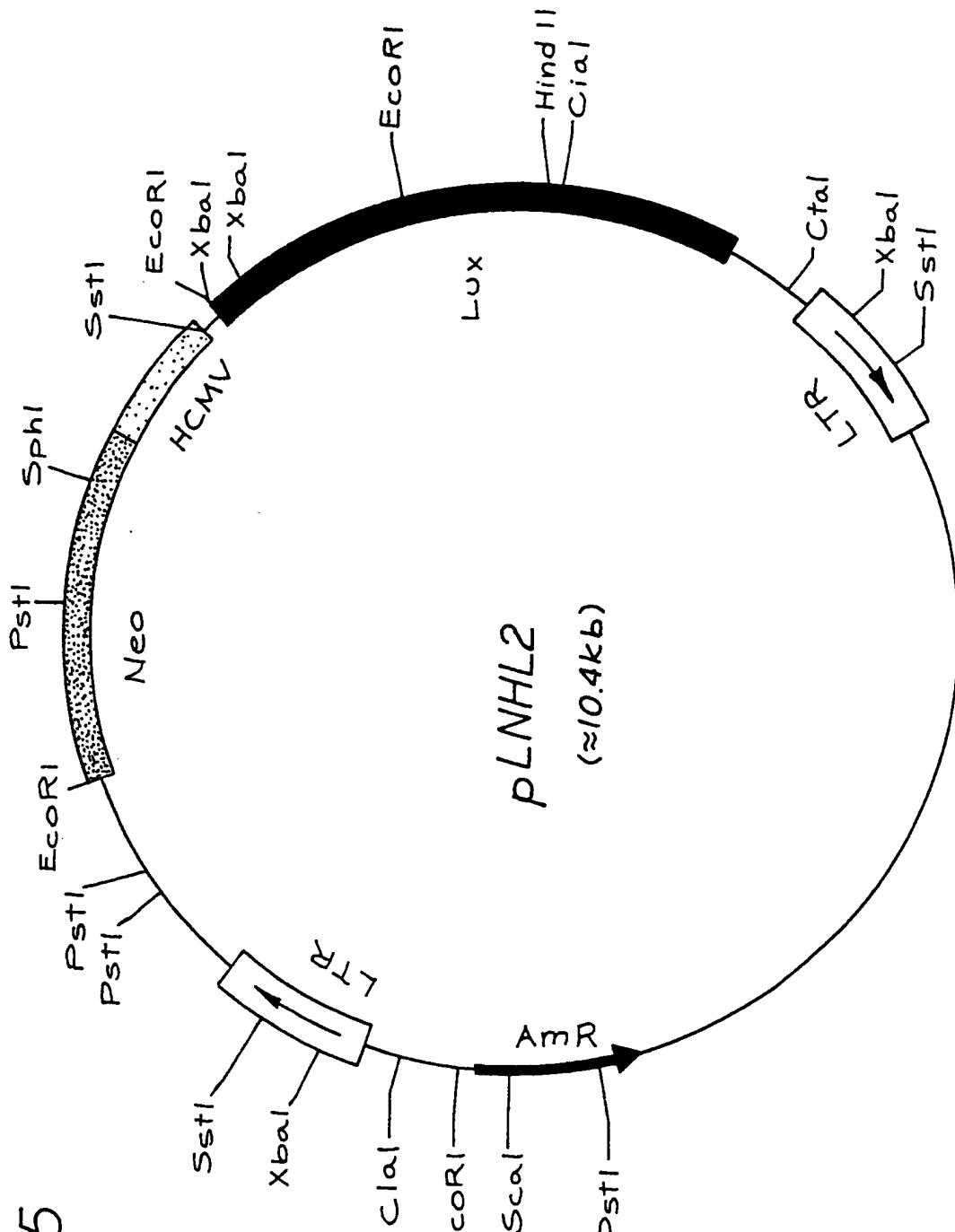
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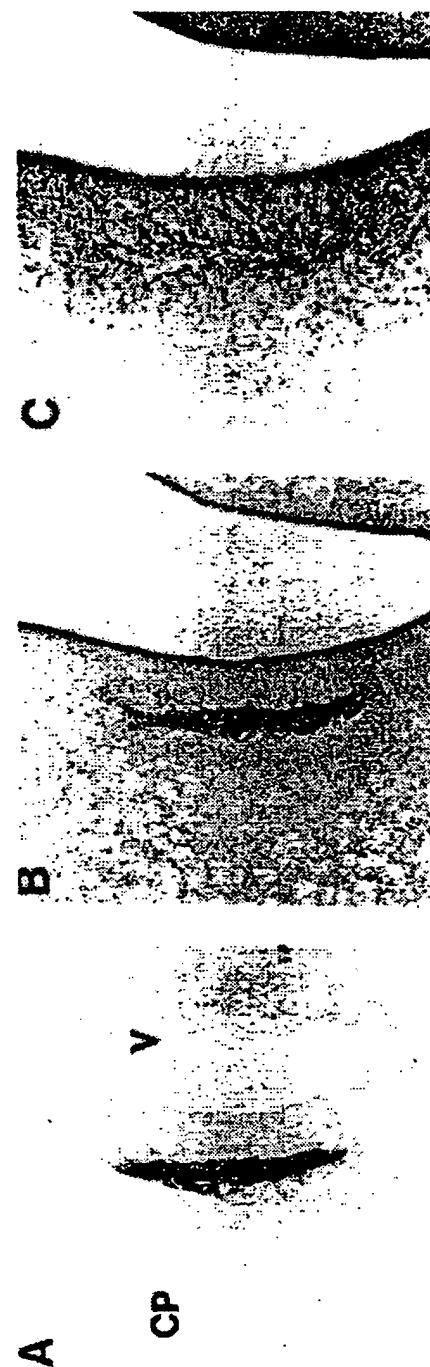
FIG. 6a.

FIG. 6b.

FIG. 6c.
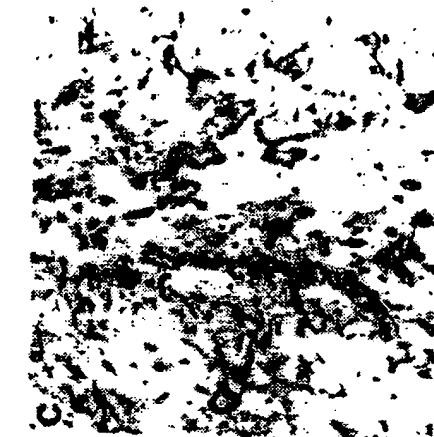
FIG. 6f.

FIG. 6e.

FIG. 6d.

FIG. 7.

FIG. 8

FIG. 9

SUBSTITUTE SHEET

FIG. 10

SUBSTITUTE SHEET

FIG. II
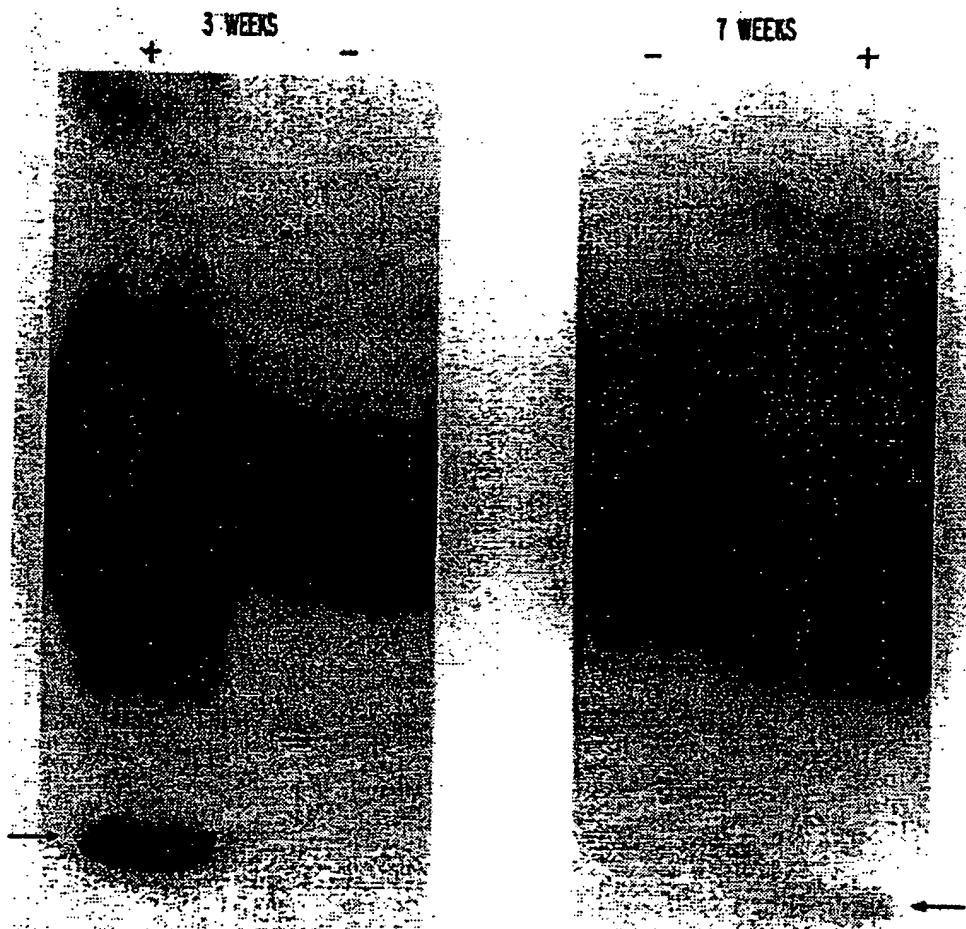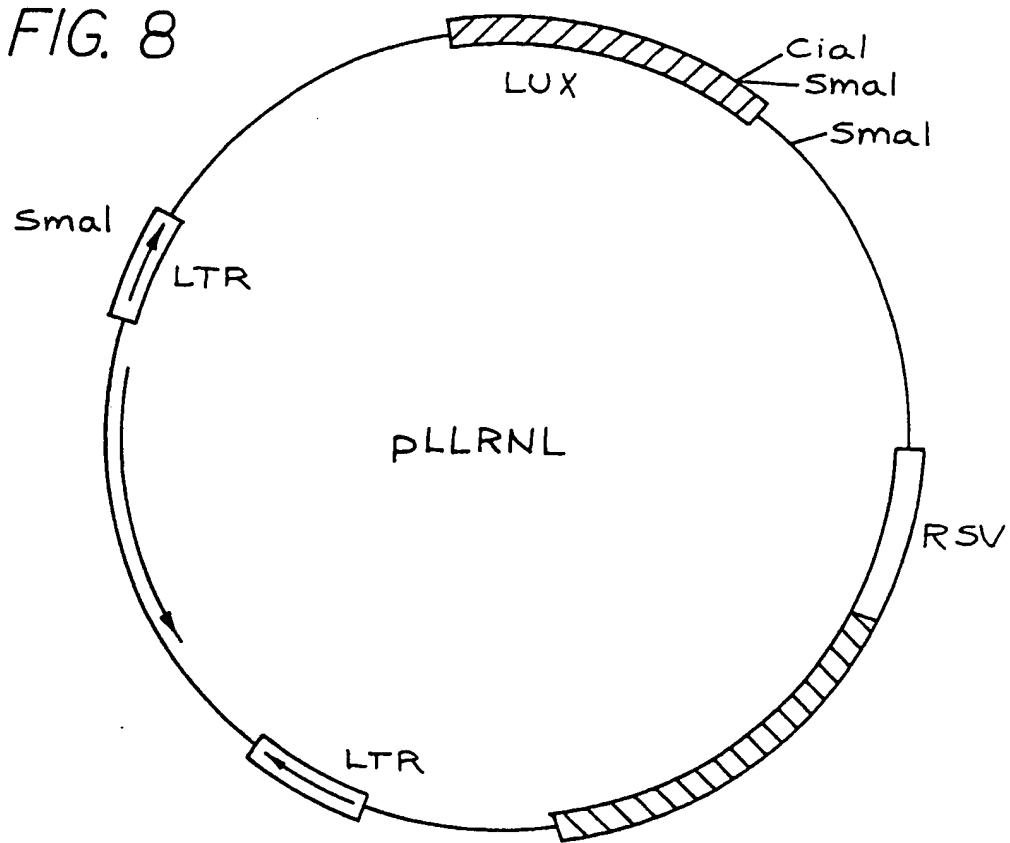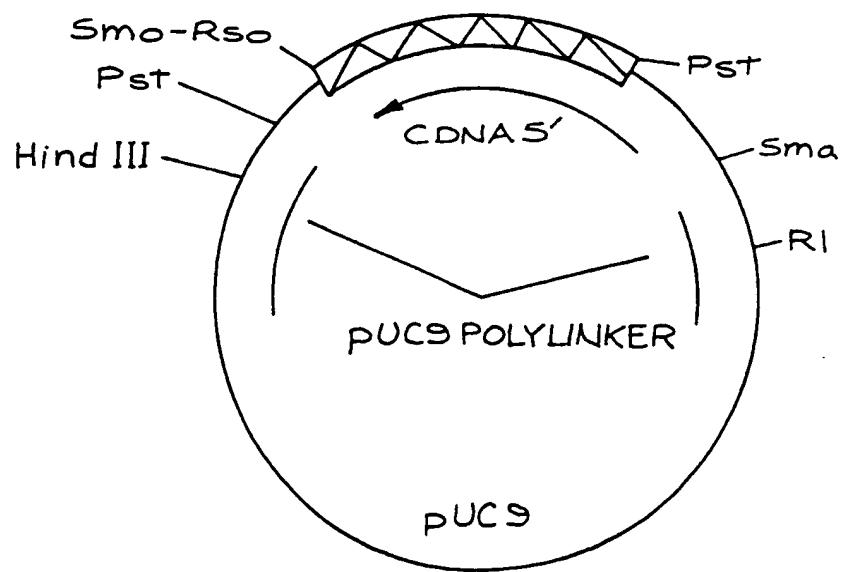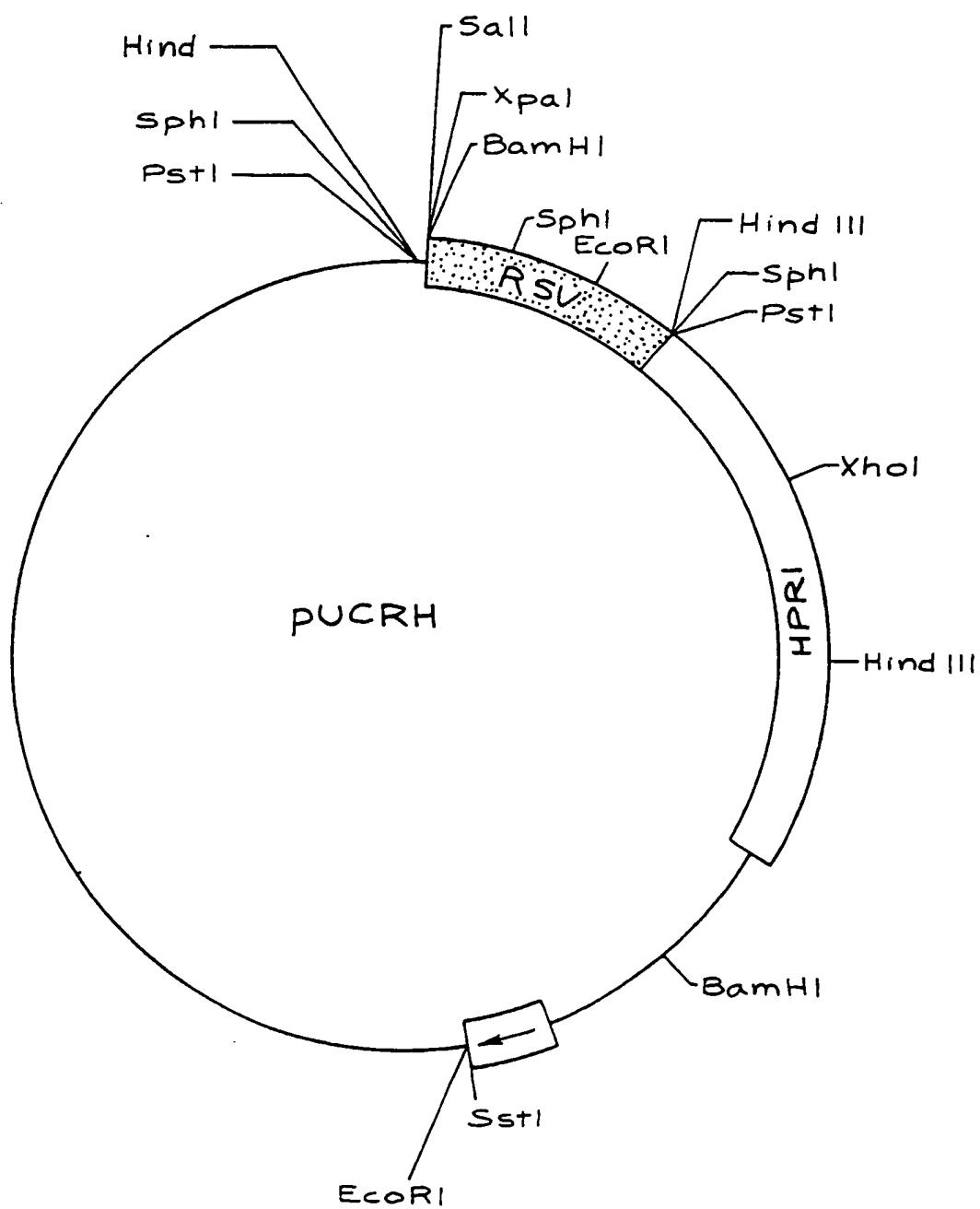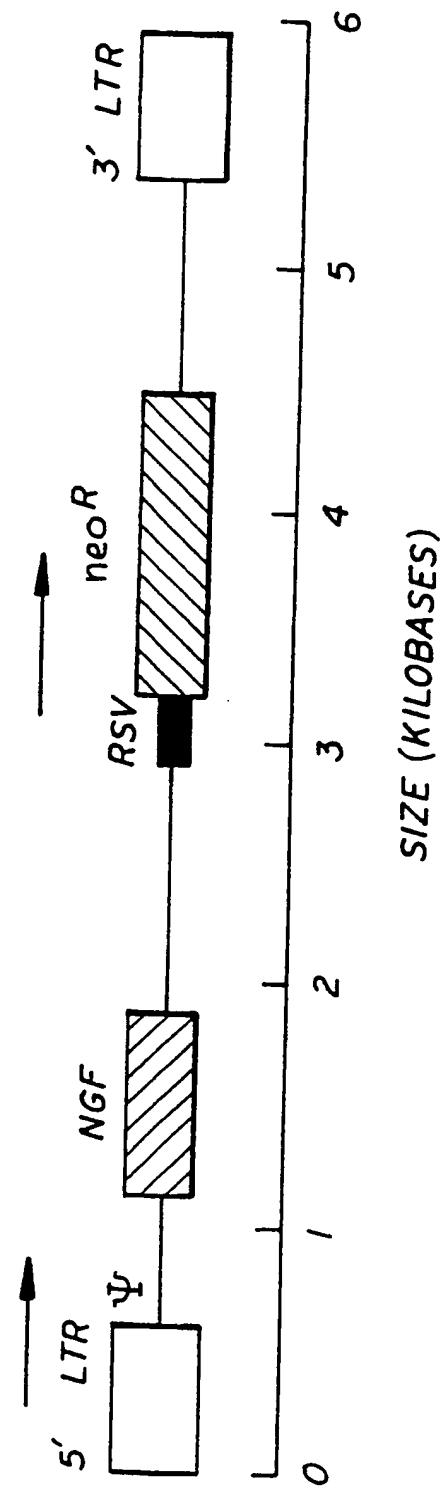
FIG. 12
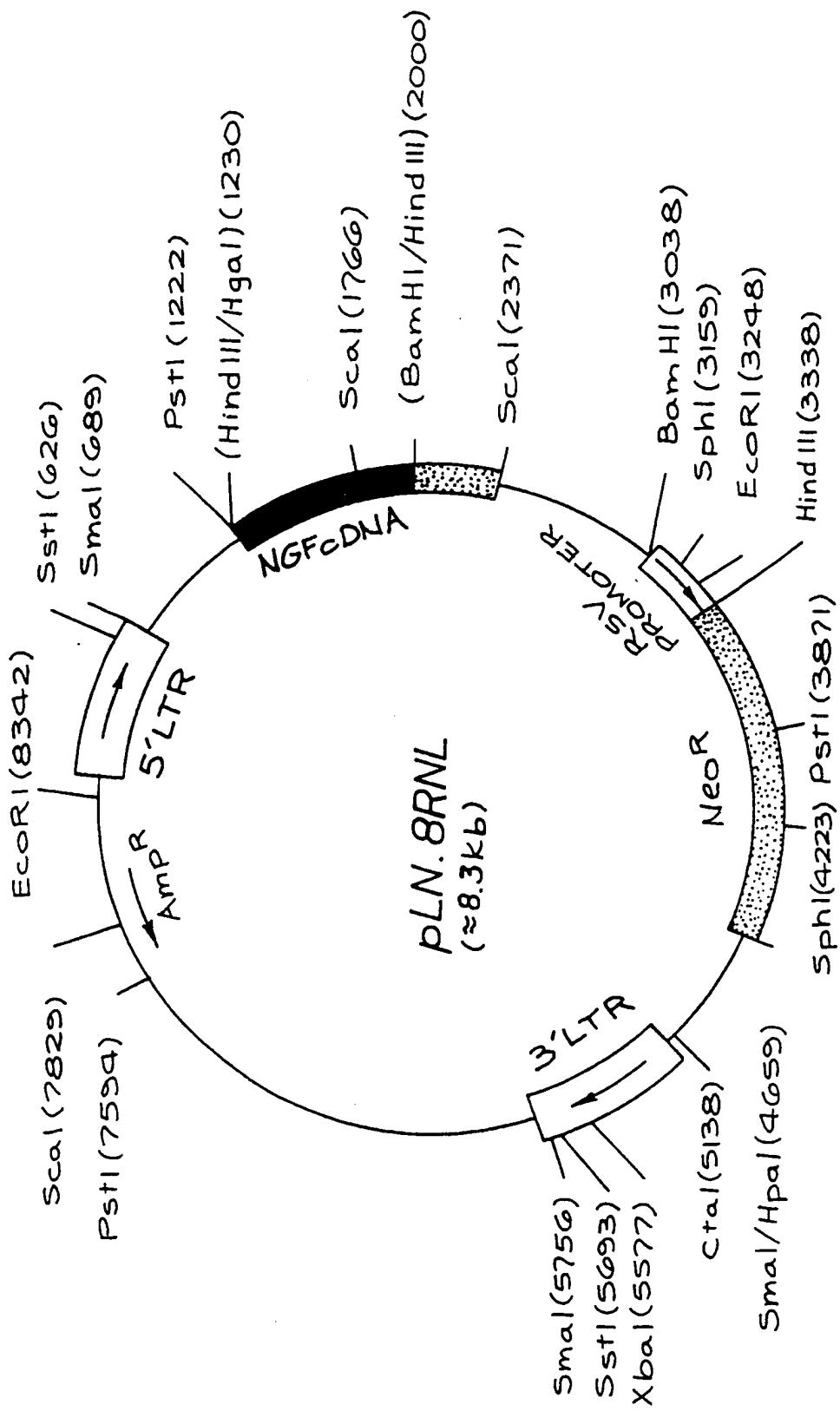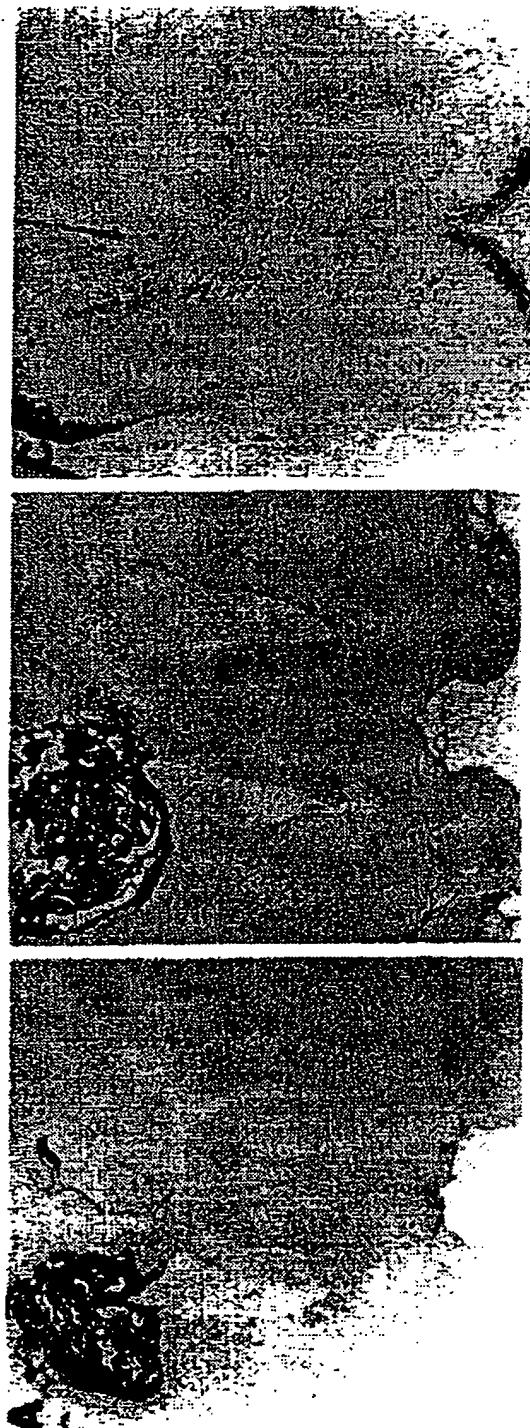
SUBSTITUTE SHEET

FIG. 13c.
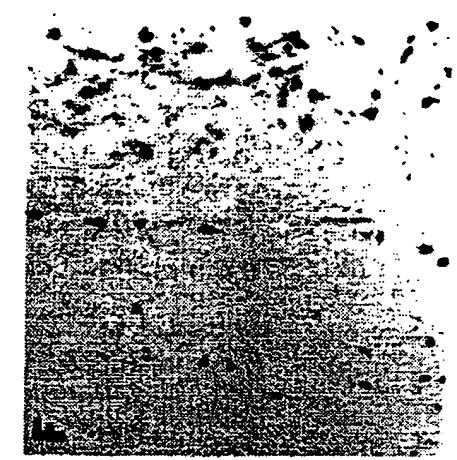
FIG. 13b.
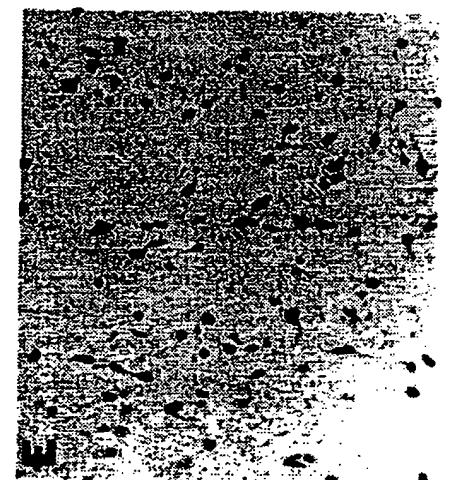
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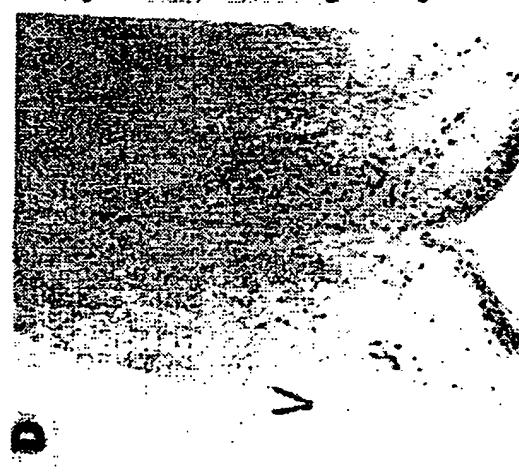
FIG. 13f.

FIG. 13e.

FIG. 13d.

FIG. 14

EFFECT OF GRAFTED NGF-PRODUCING FIBROBLAST
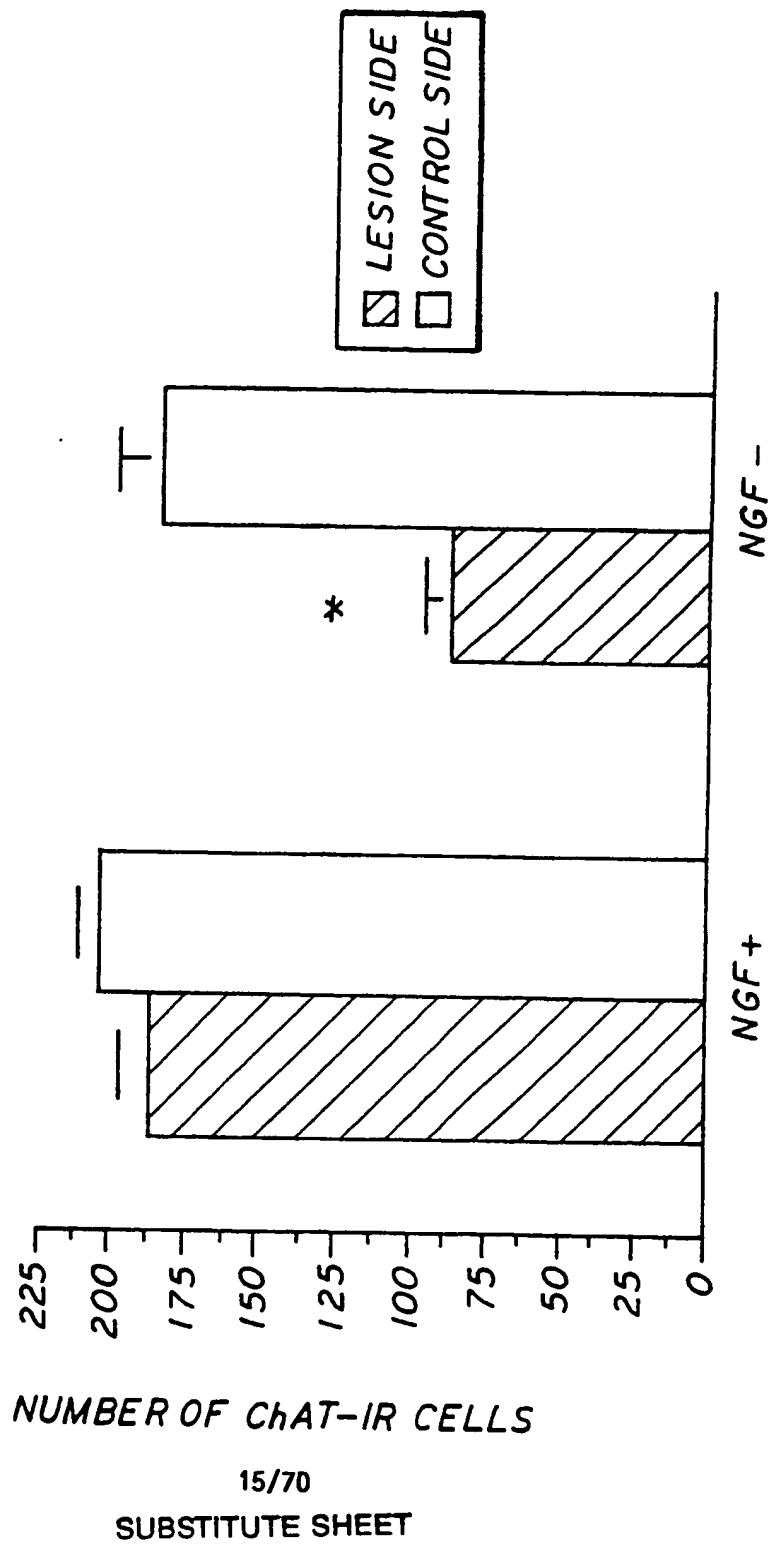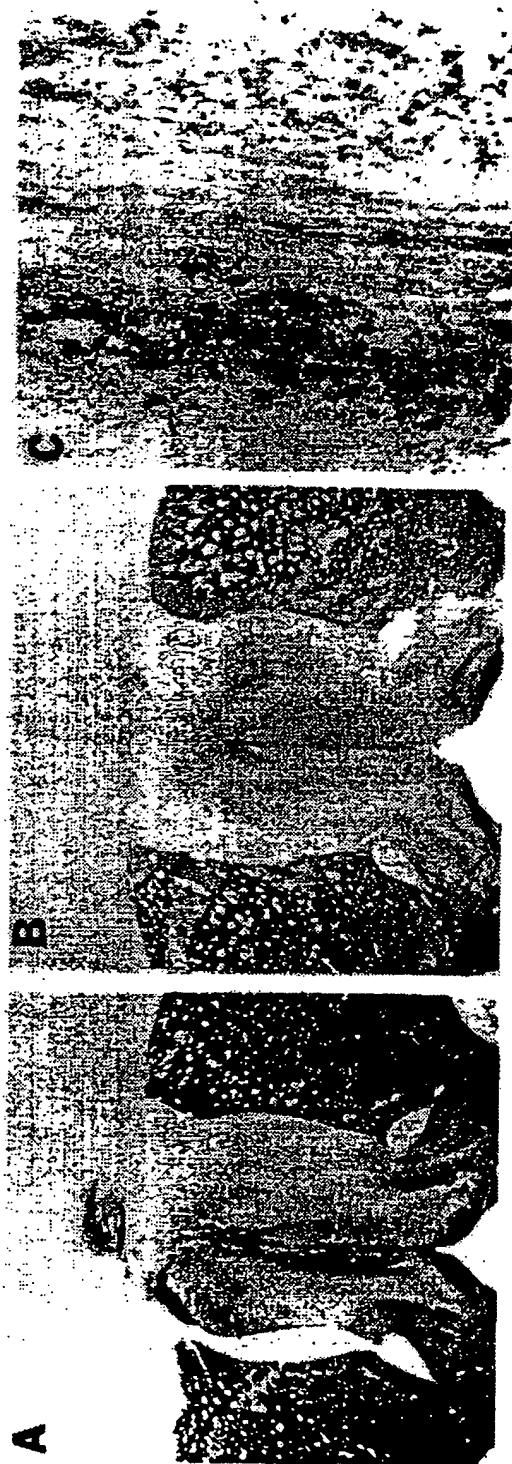
FIG. 15c.

FIG. 15b.
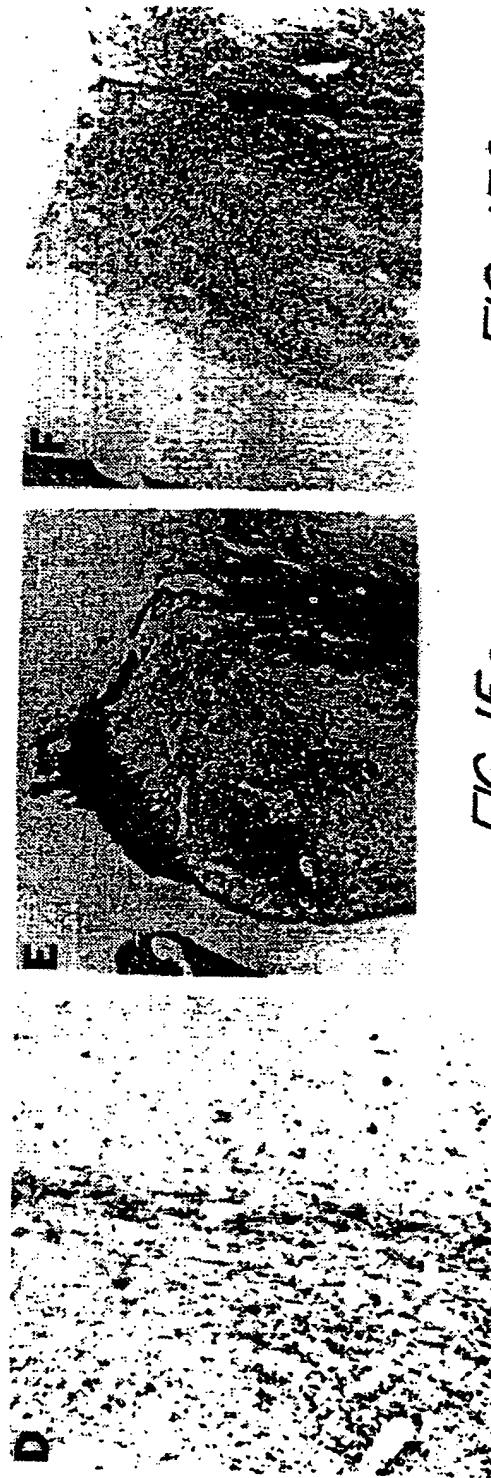
FIG. 15a.

FIG. 15f.

FIG. 15e.

FIG. 15d.

FIG. 16

LThRNL
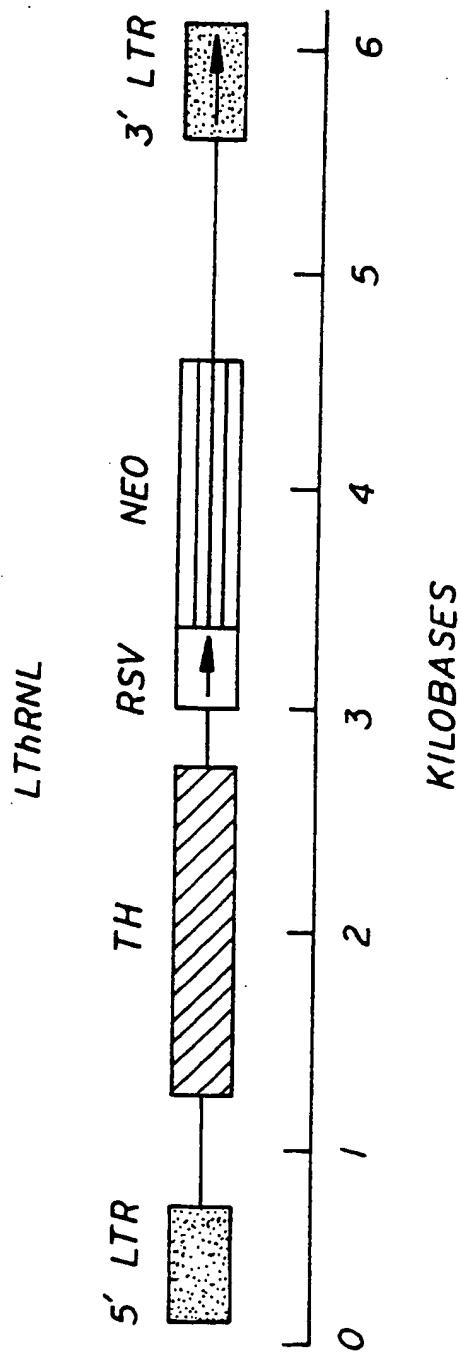
FIG. 17
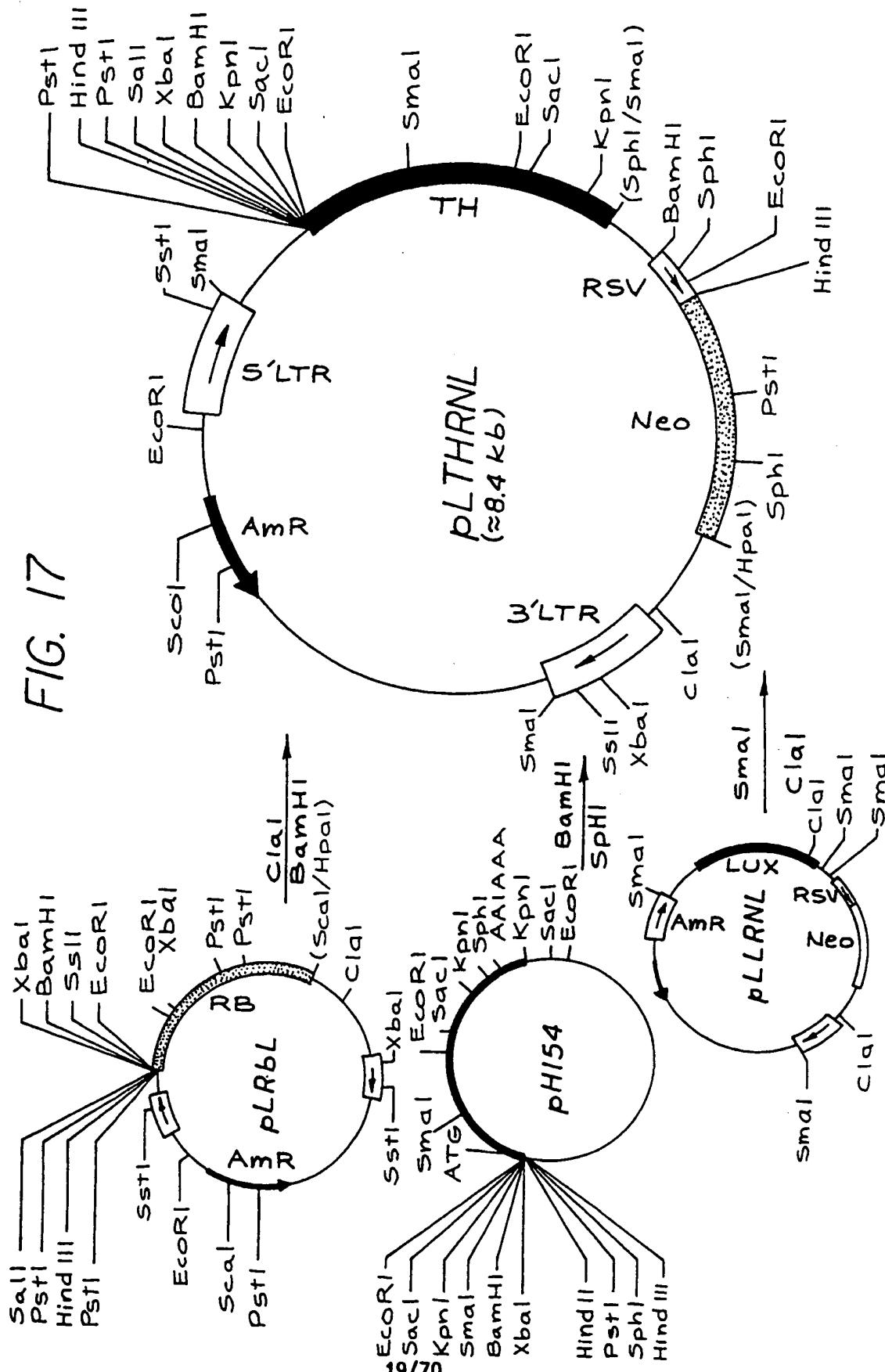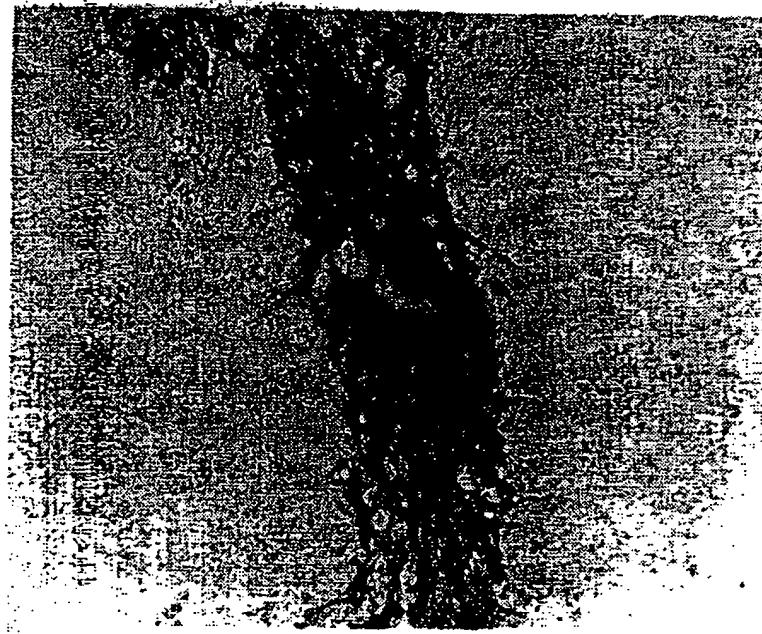
FIG. 18a.

FIG. 18b.

FIG. 18c.
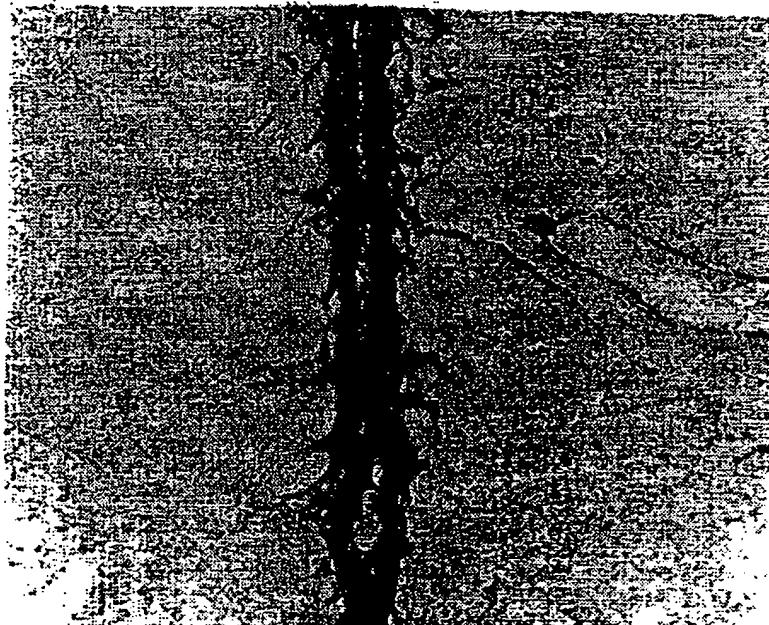
FIG. 18d.

ROTATIONAL ASSYMETRY
FOLLOWING FIBROBLAST
TRANSPLANTATION
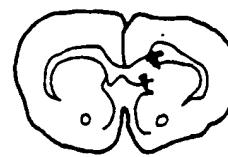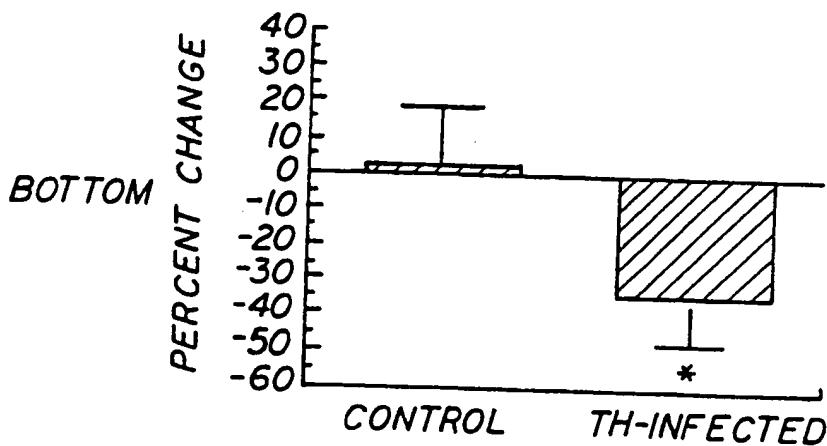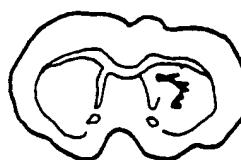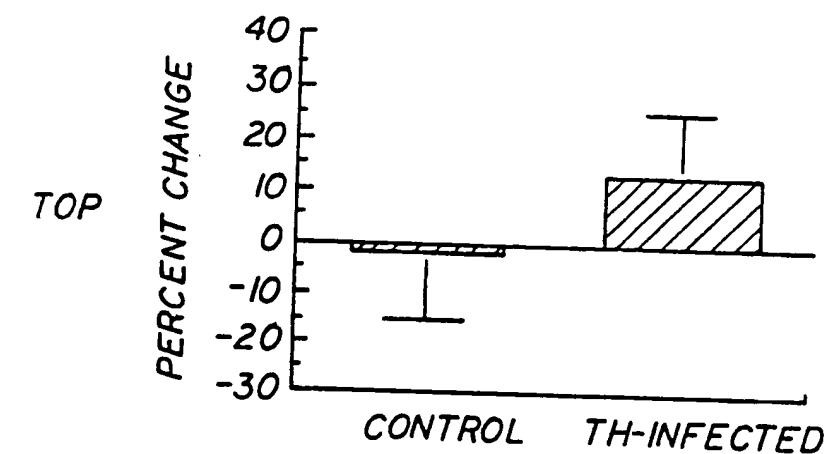
FIG. 19

FIG. 20

FIBROBLAST GROWTH CURVES

PRIMARY SKIN FIBROBLASTS
RAT-I FIBROBLASTS

DAYS IN CULTURE

CELLS COUNTED (X 1000)

10000
1000
100
10
1

0   10   20   30
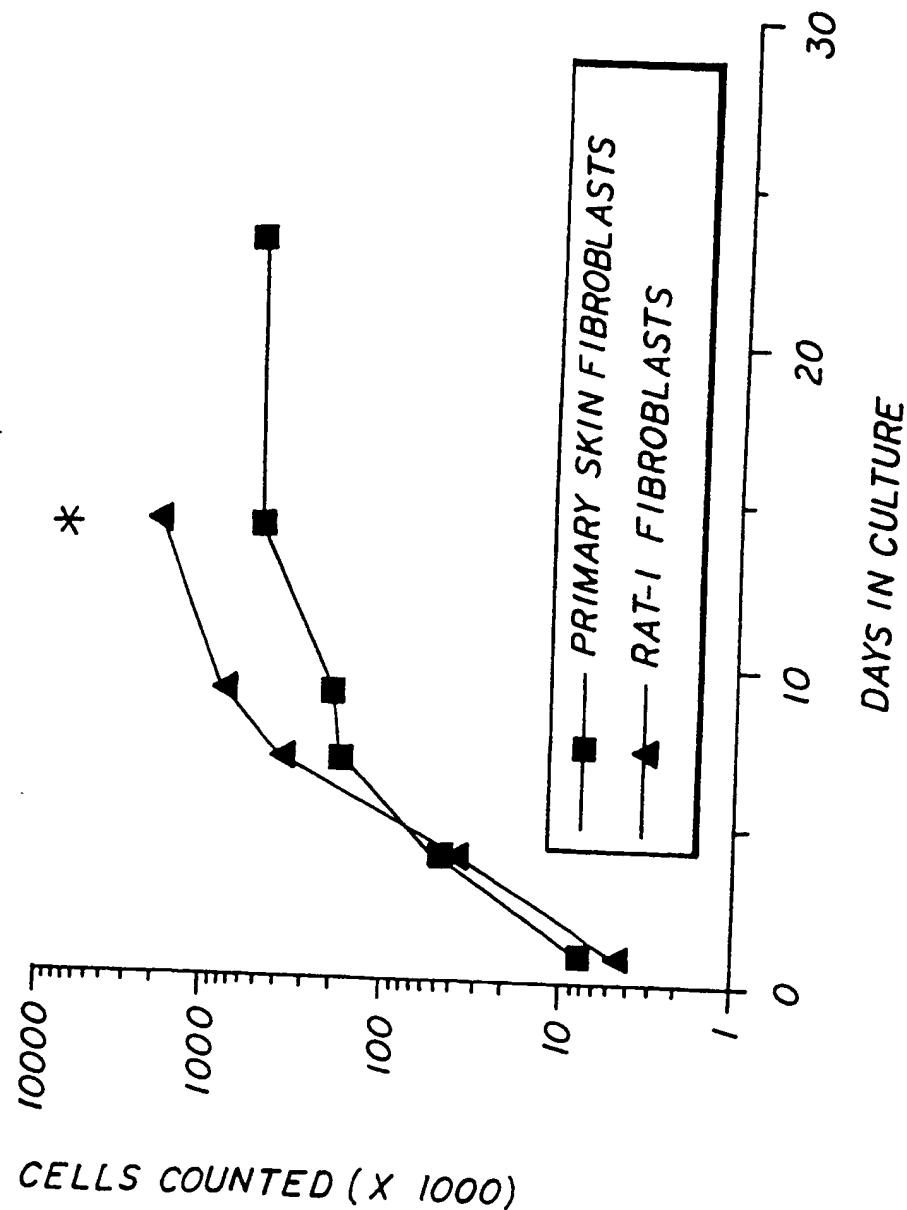
FIG. 21
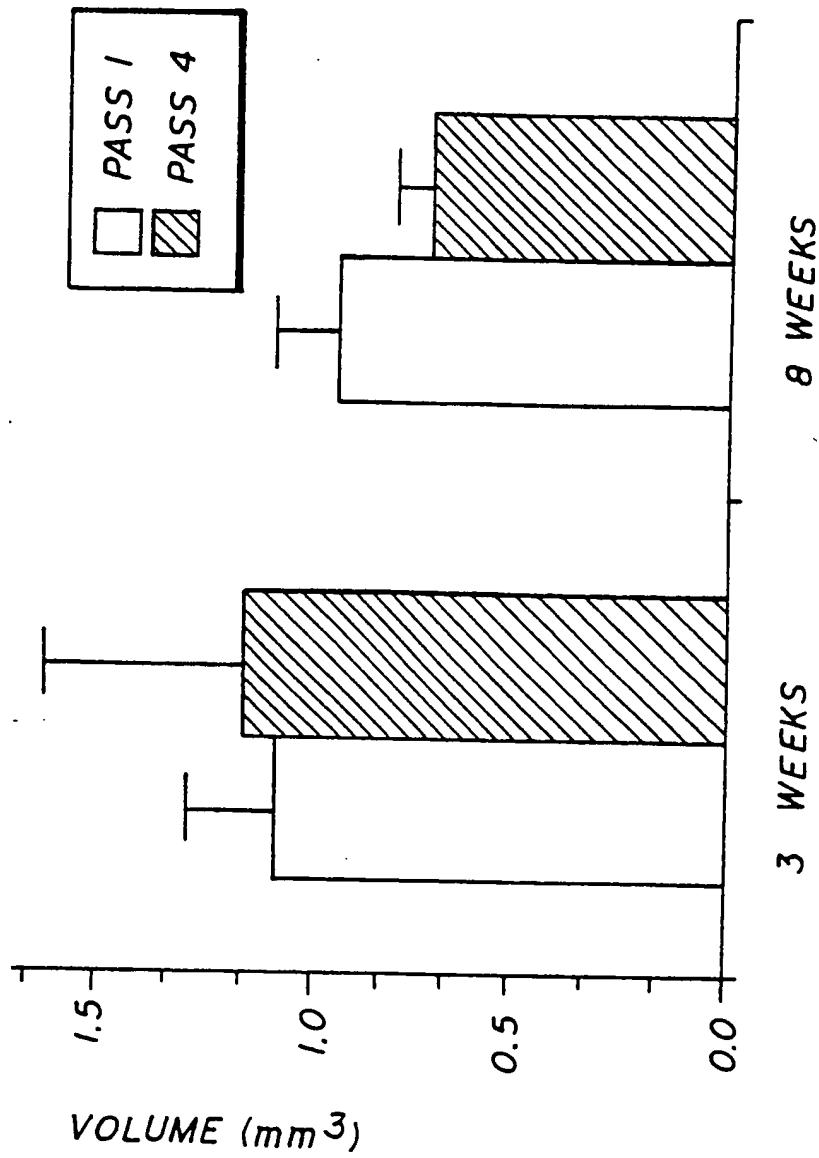
PRIMARY FIBROBLAST GRAFTS

FIG. 22

PRIMARY FIBROBLAST GRAFTS
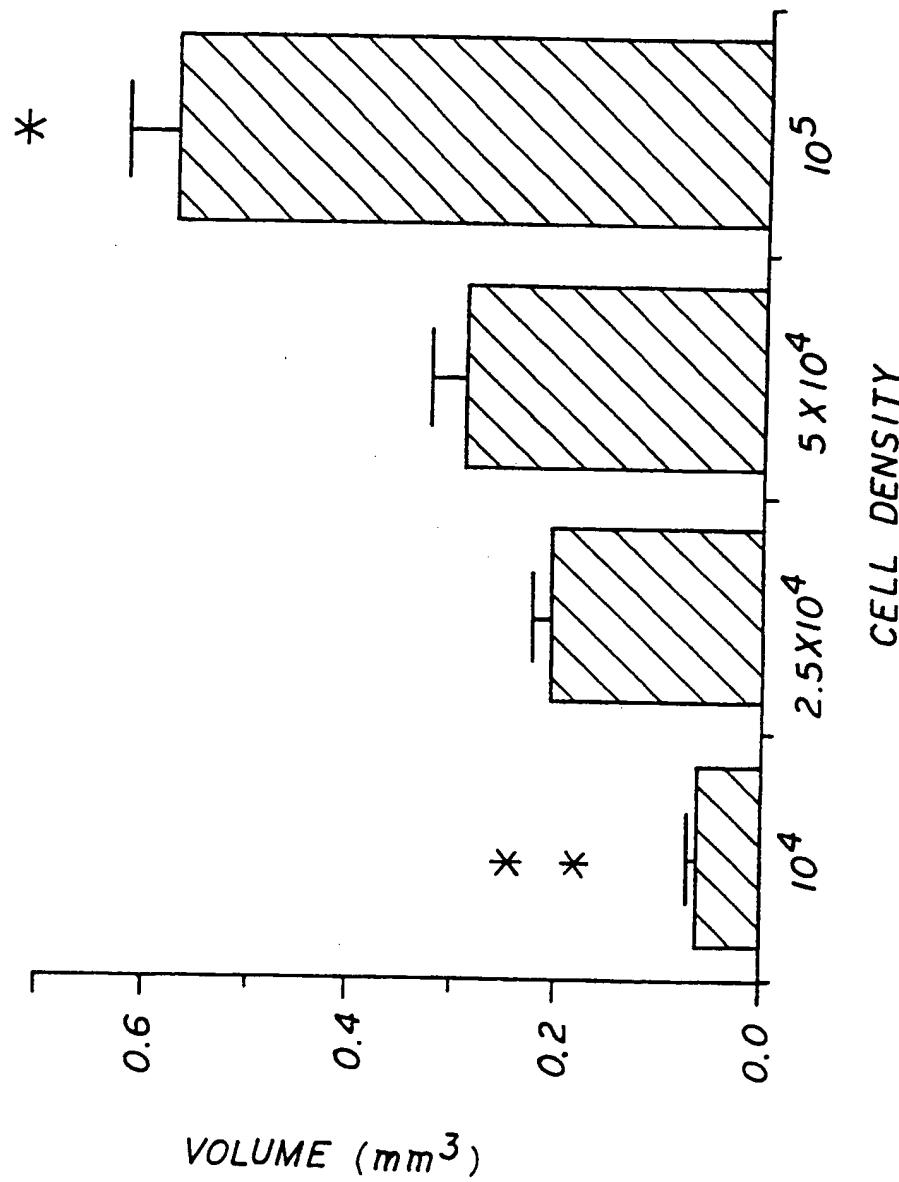
FIG.23b
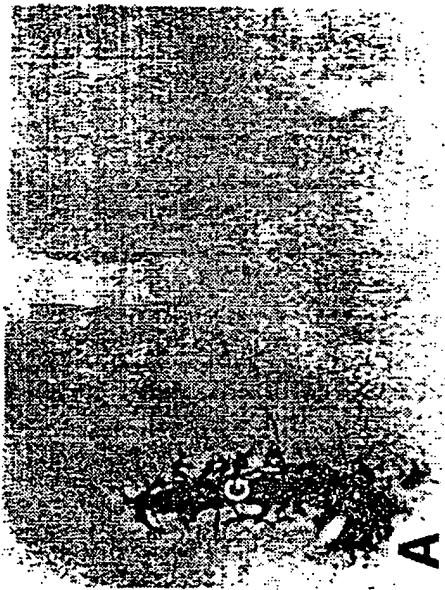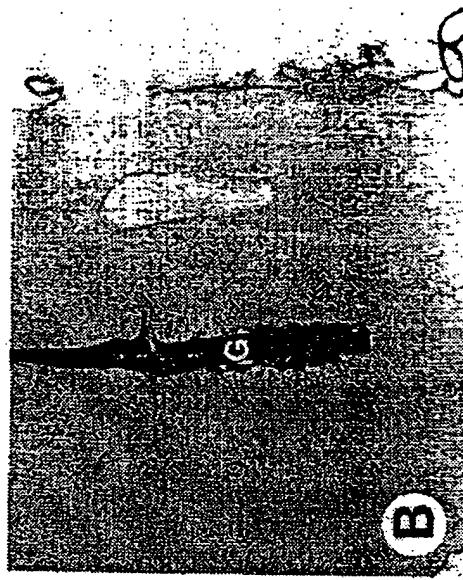
FIG.23d

FIG.23a

FIG.23c
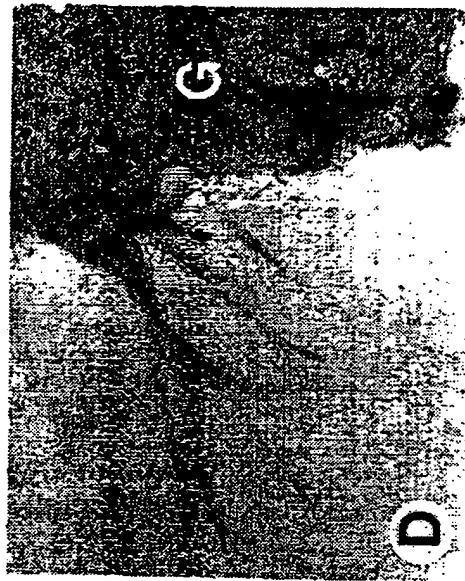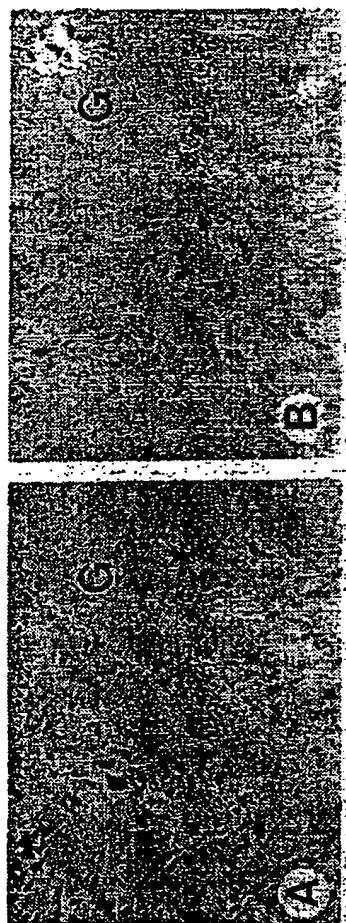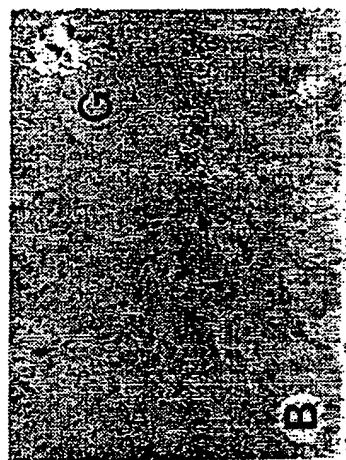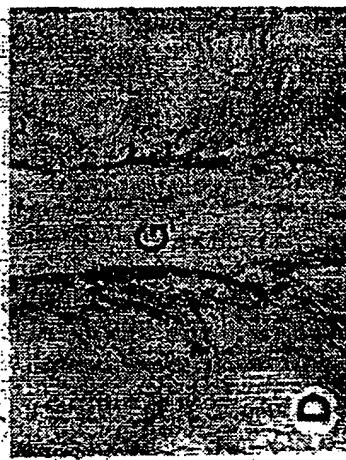
FIG.24a   FIG.24b   FIG.24c   FIG.24d   FIG.24e   FIG.24f
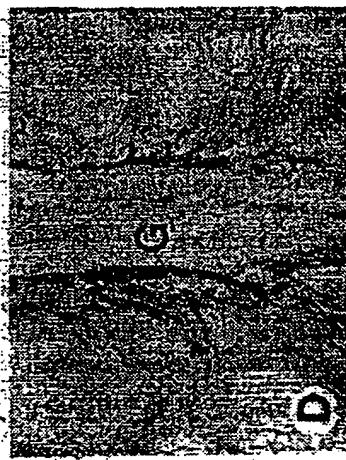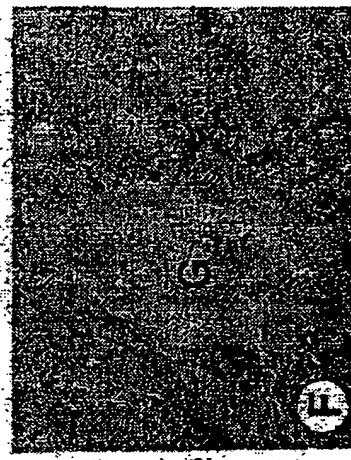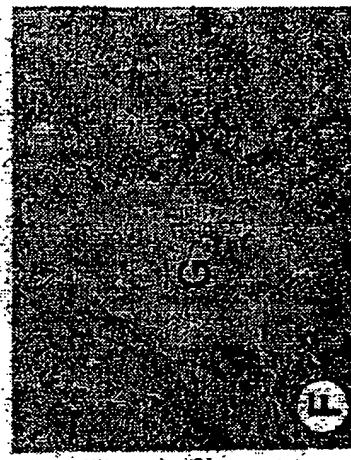
FIG. 25a
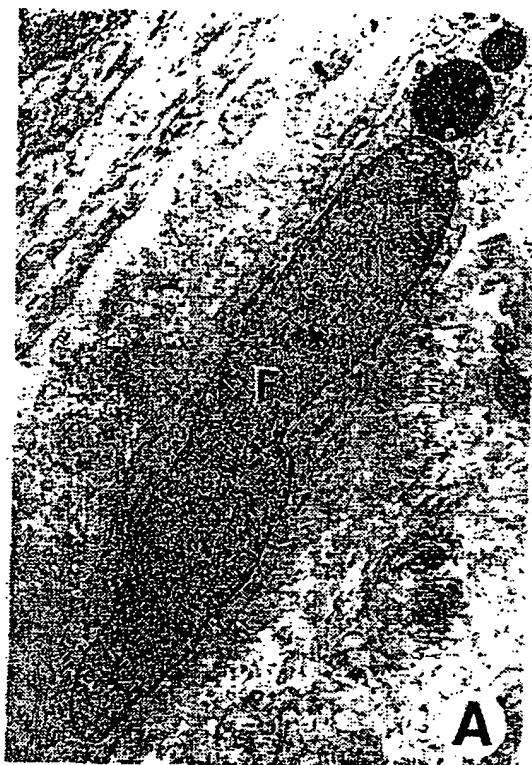
FIG. 25b

FIG. 25c

FIG. 26a
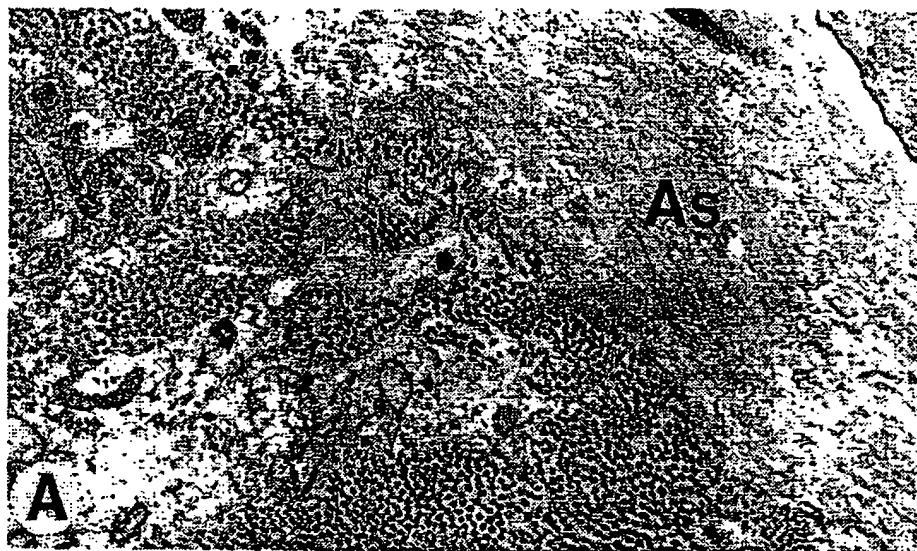
FIG. 26b

FIG. 27a
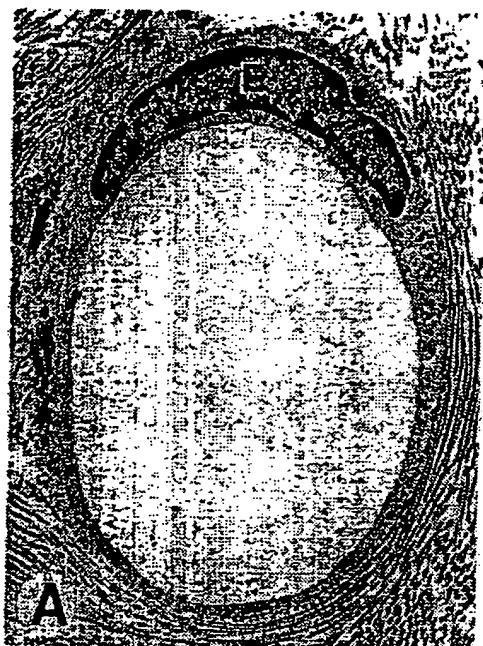
FIG. 27b
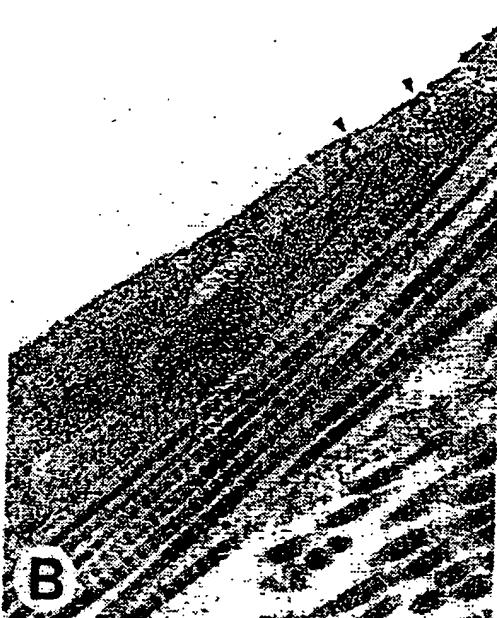
FIG. 27c
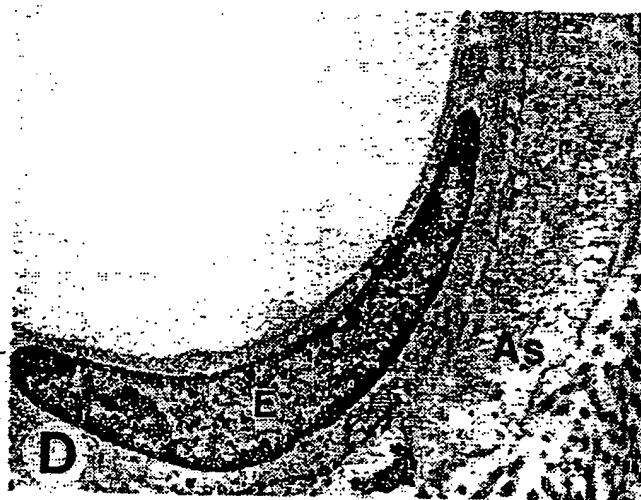
FIG. 27d

FIG. 28

31/70

FIG. 29a

TOP

CONTROL

FIG. 29b

MIDDLE

FF2/BGAL

FIG. 29c

BOTTOM

FF2/TH

SUBSTITUTE SHEET

FIG. 30a

FIG. 30b

SUBSTITUTE SHEET
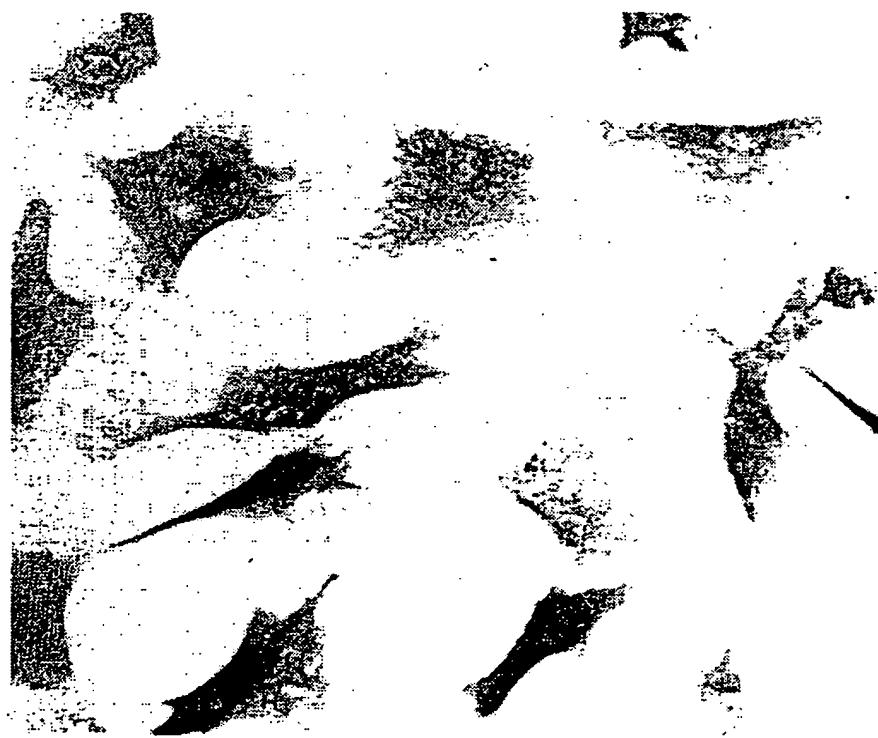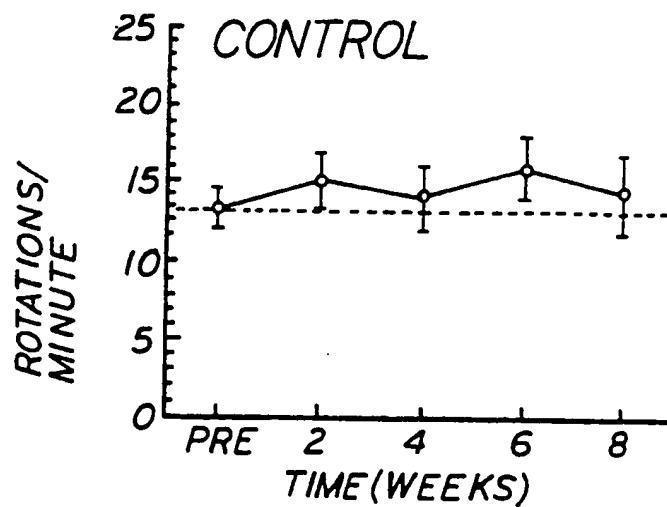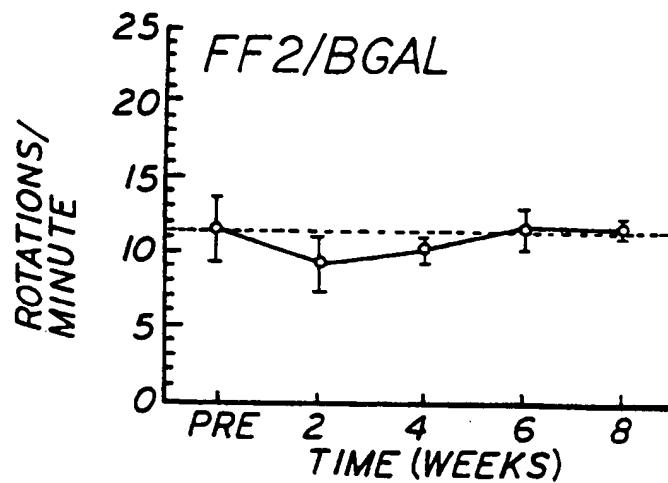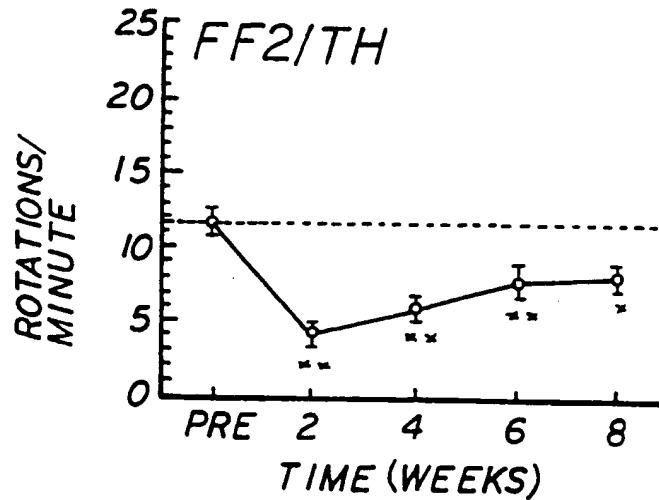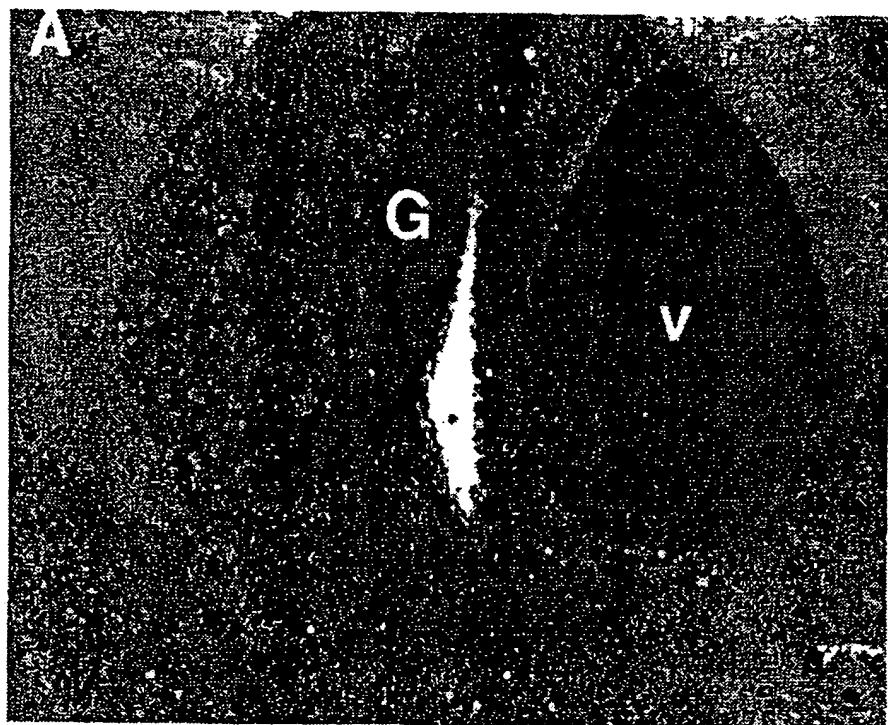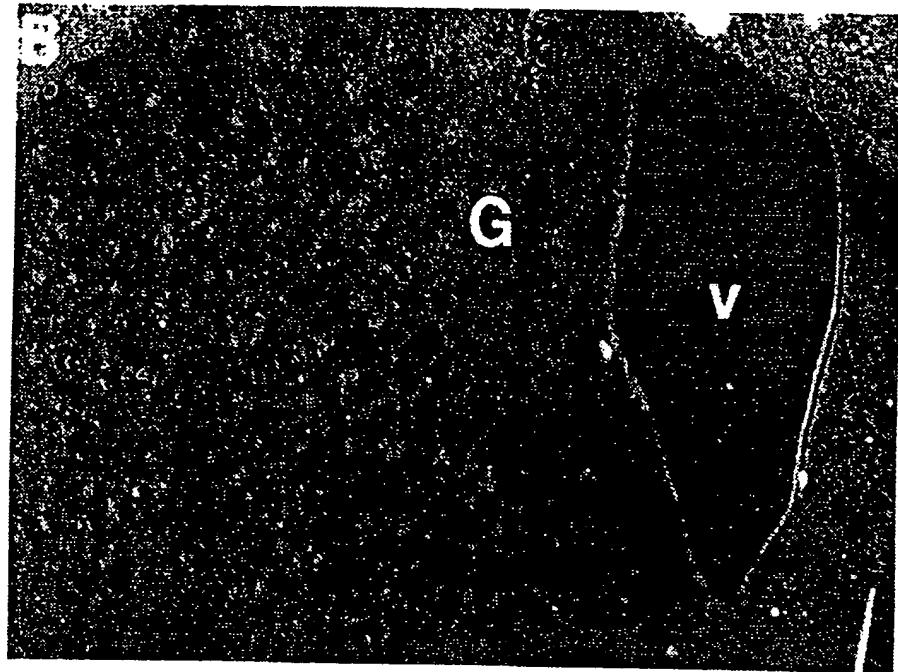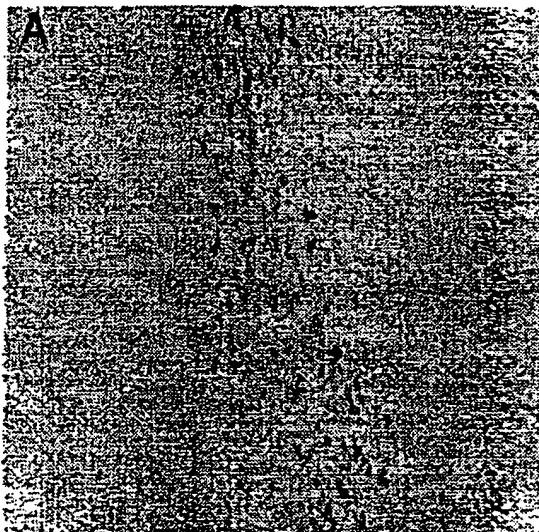
FIG. 31a

FIG. 31b

FIG. 31c

FIG. 31d

SUBSTITUTE SHEET
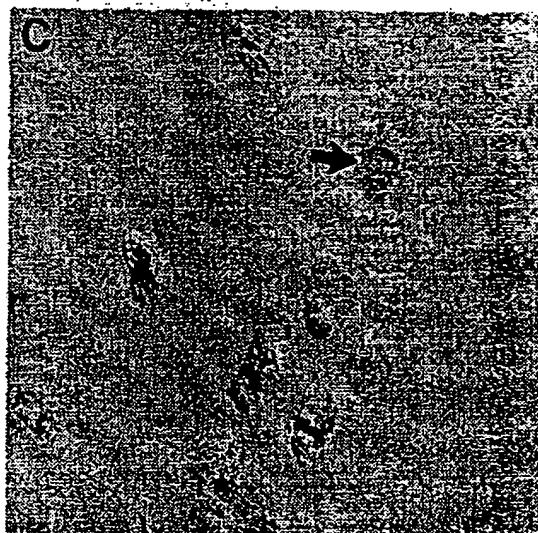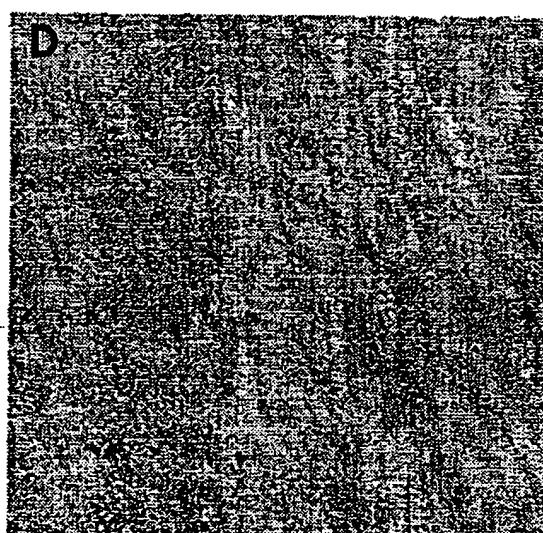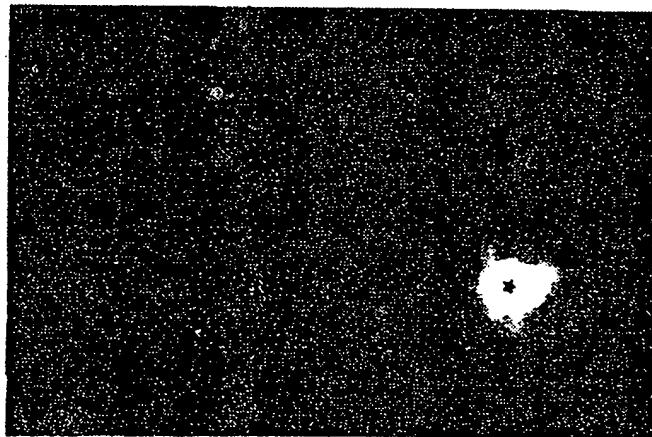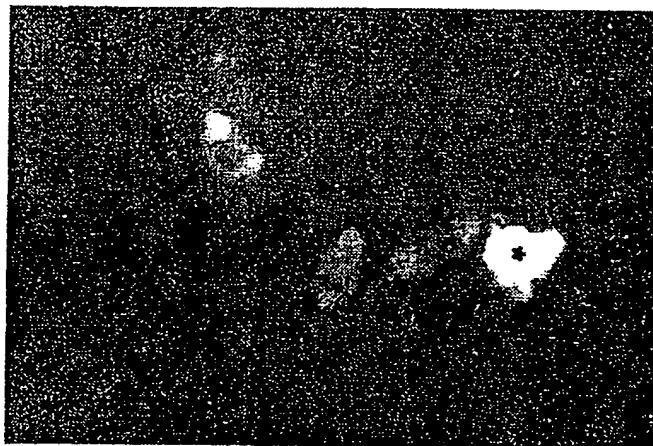
FIG. 32a

FIG. 32b

FIG. 33

NGF SECRETION IN VITRO

DAY 1 (33%)
DAY 2
DAY 3
DAY 5 (100%)
DAY 16

NGF (ng/10^6 CELLS/HOUR)

12.0 10.0 8.0 6.0 4.0 2.0 0.0

hRAT   hnHUMAN hMONKEY haHUMAN mRAT

GENE AND CELL TYPE

FIG. 34

5'LTR   dChAT   RSV   NEO   3'LTR

I KILOBASE

RELATIVE ChAT
ACTIVITY

FIG.36b

FIG.36c

RELATIVE ACh
CONTENT

SUBSTITUTE SHEET
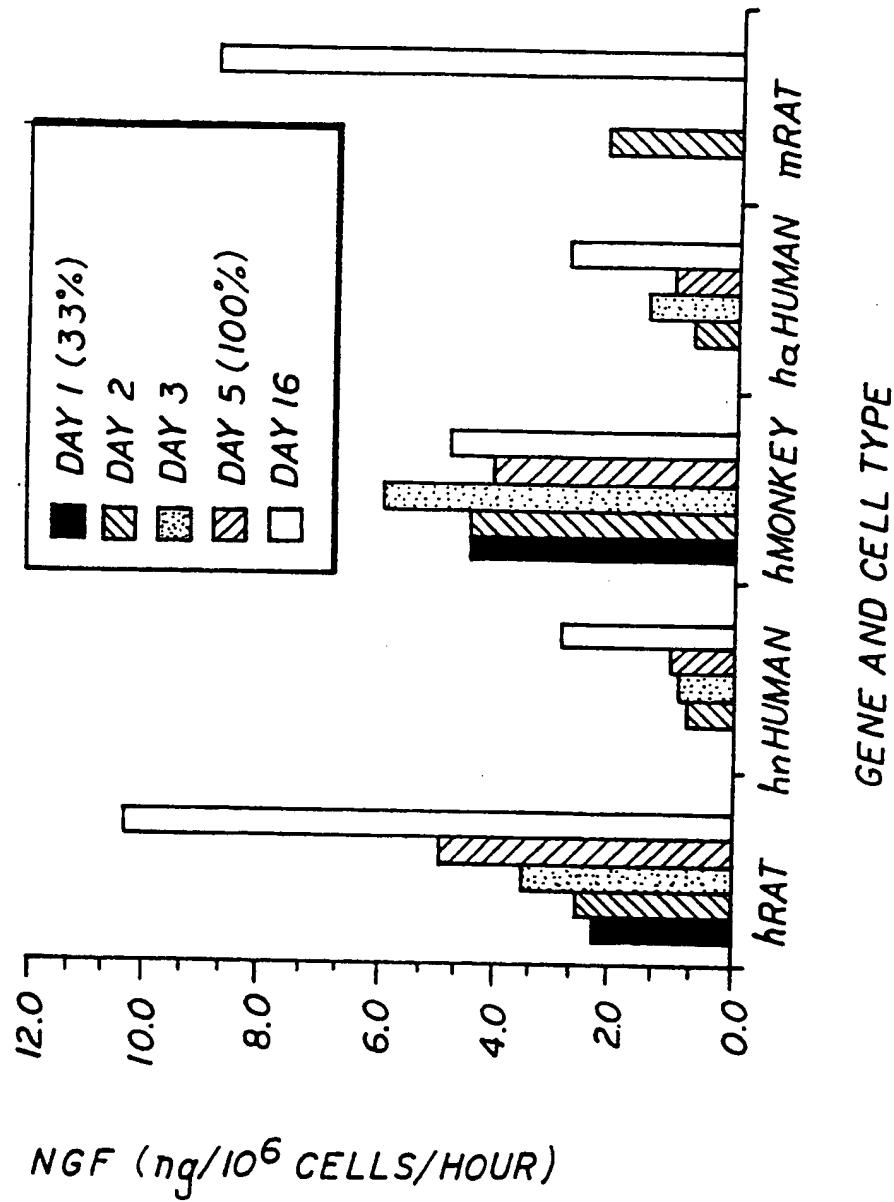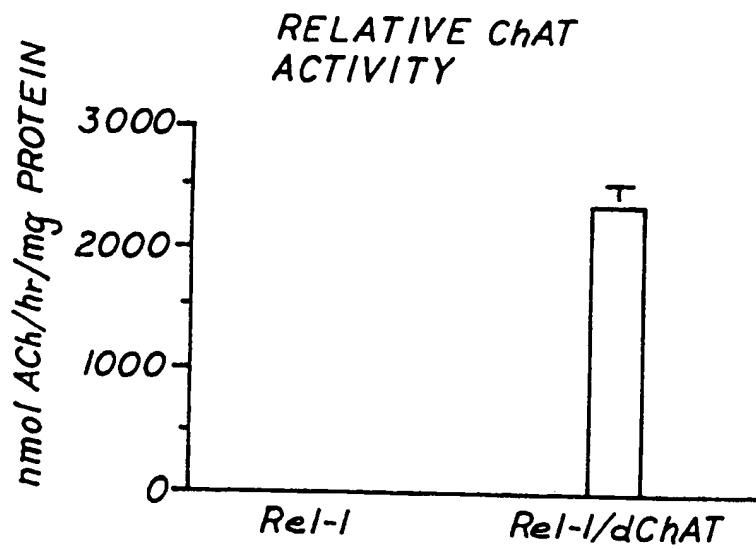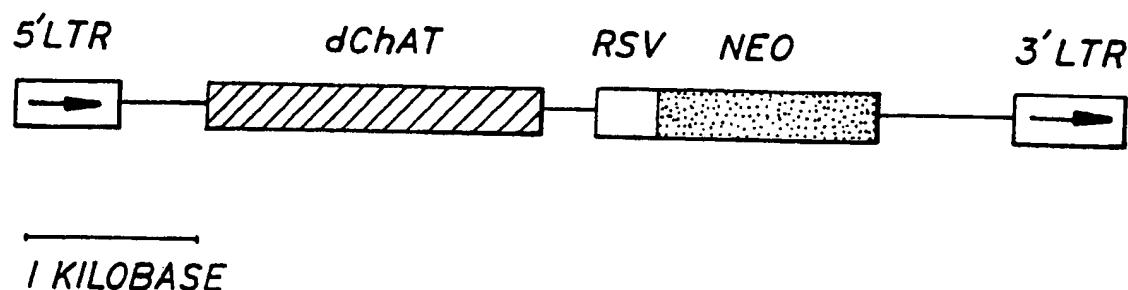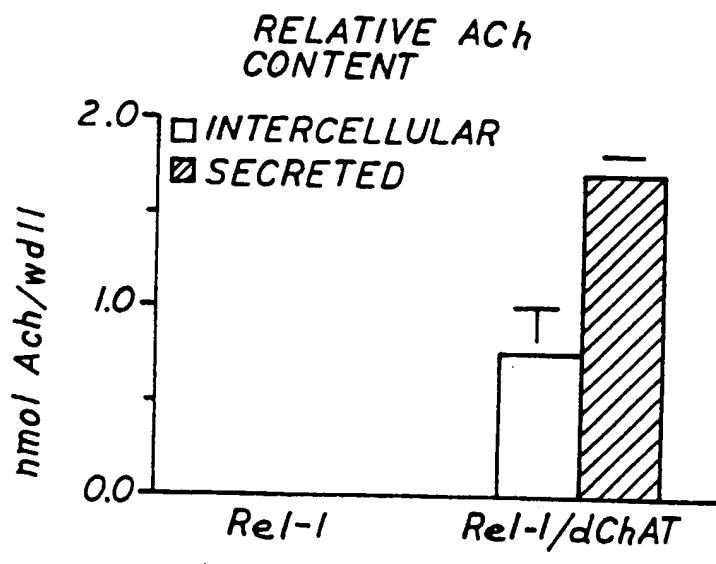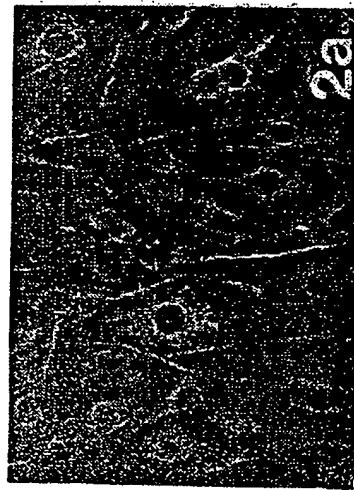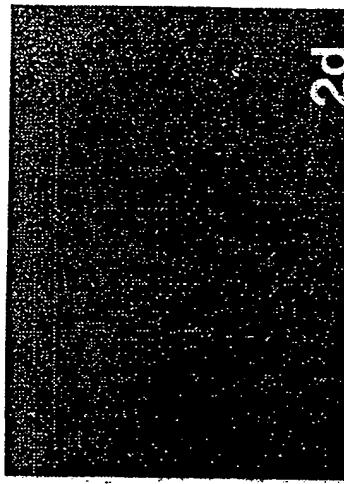
FIG.35d

FIG.35e

FIG.35f

FIG.35a

FIG.35b

FIG.35c
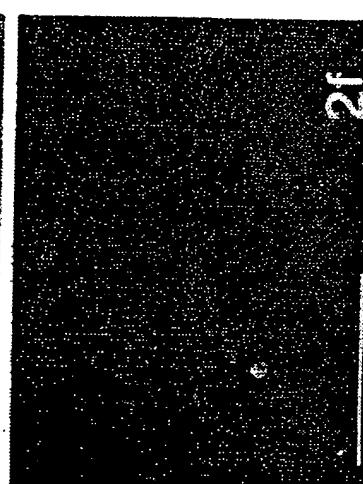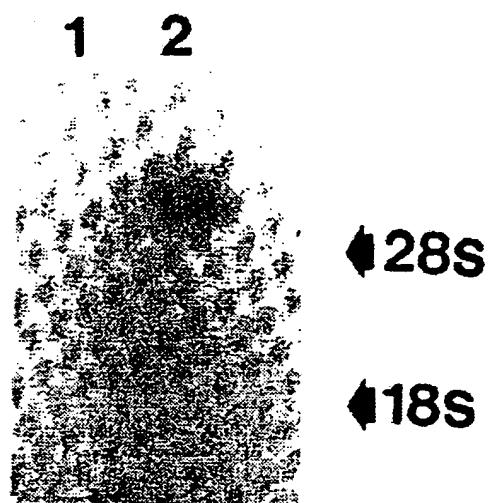
FIG. 36a

FIG.37a

EFFECT OF CHOLINE ON Ach
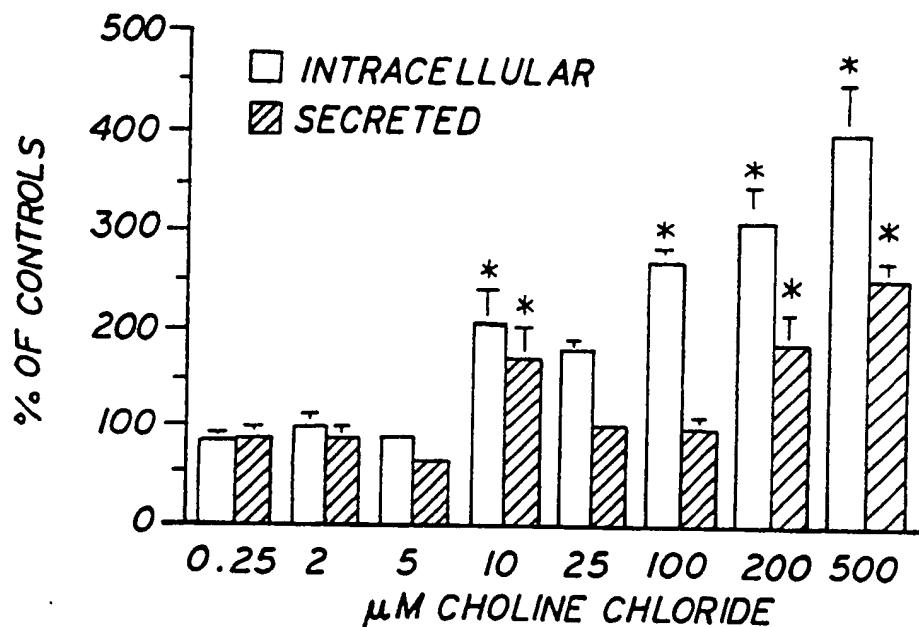
FIG.37b

EFFECT OF CHOLINE ON ChAT ACTIVITY

FIG. 38a

EFFECT OF SERUM STARVATION
ON ChAT ACTIVITY

FIG. 38c

FIG. 38b

FIG. 39a

EFFECT OF CONFLUENCE ON ChAT ACTIVITY

(Bar graph: y-axis "nmol Ach/hr/mg PROTEIN" from 0 to 3000, x-axis "DAYS OF POST-CONFLUENCE" at 0, 3, 7, 14)

FIG. 39c

(Bar graph: y-axis "RATIO dChAT/CYCLO." from 0.0 to 0.4, x-axis categories LOG, 3 DPC, 14 DPC)

SUBSTITUTE SHEET

FIG. 39b

FIG. 40
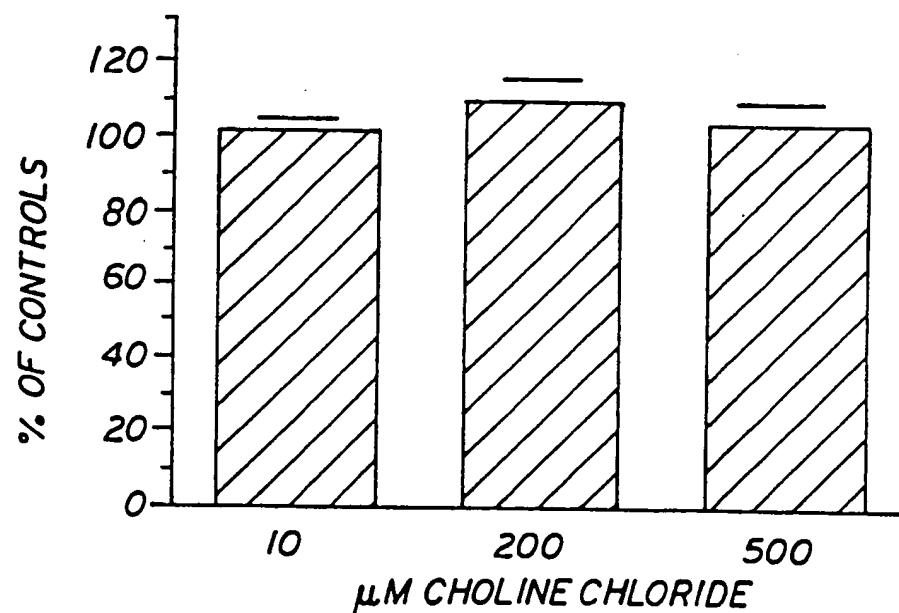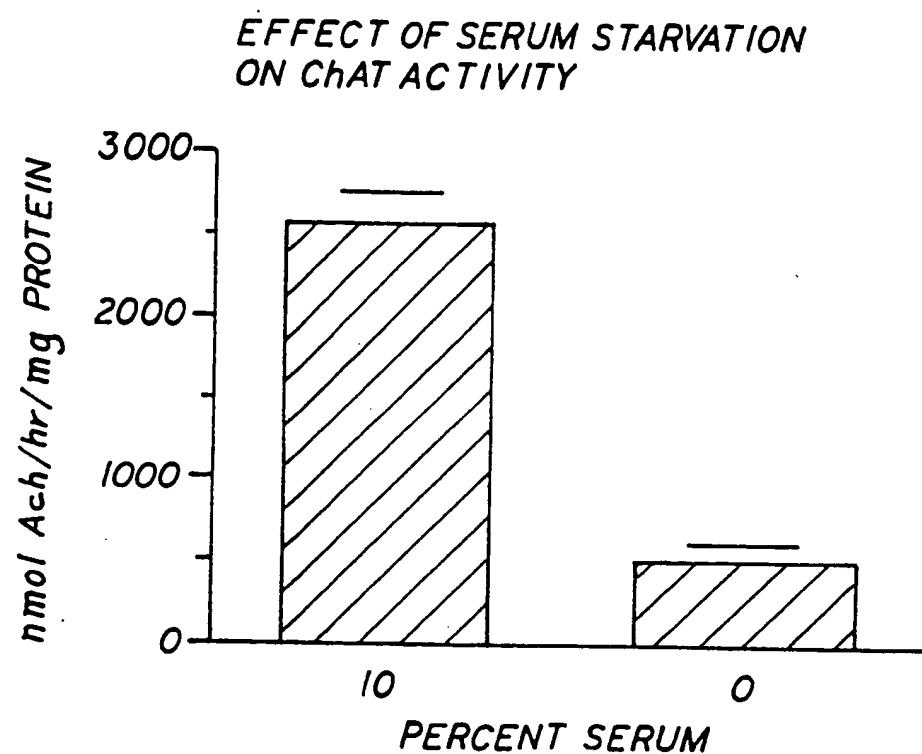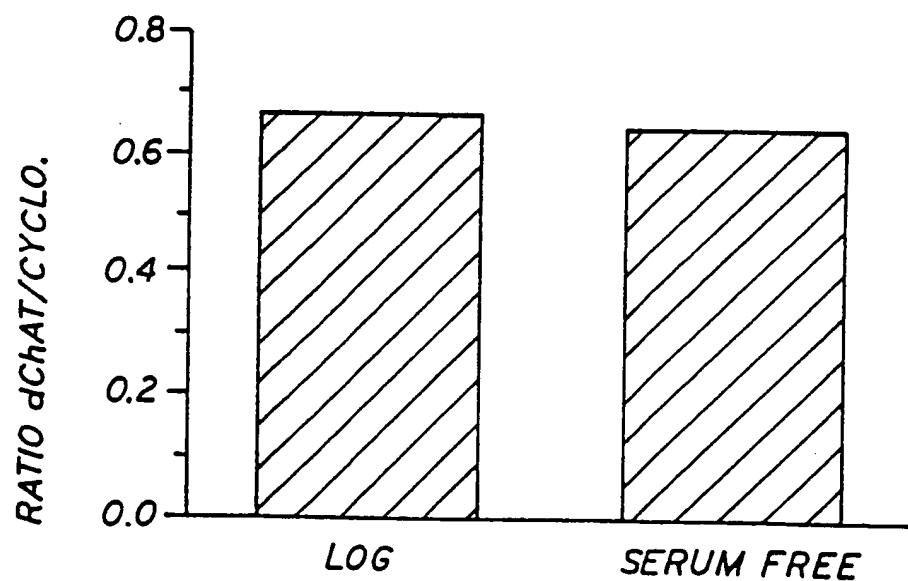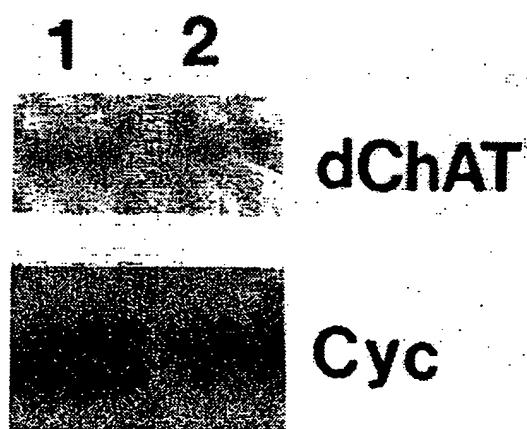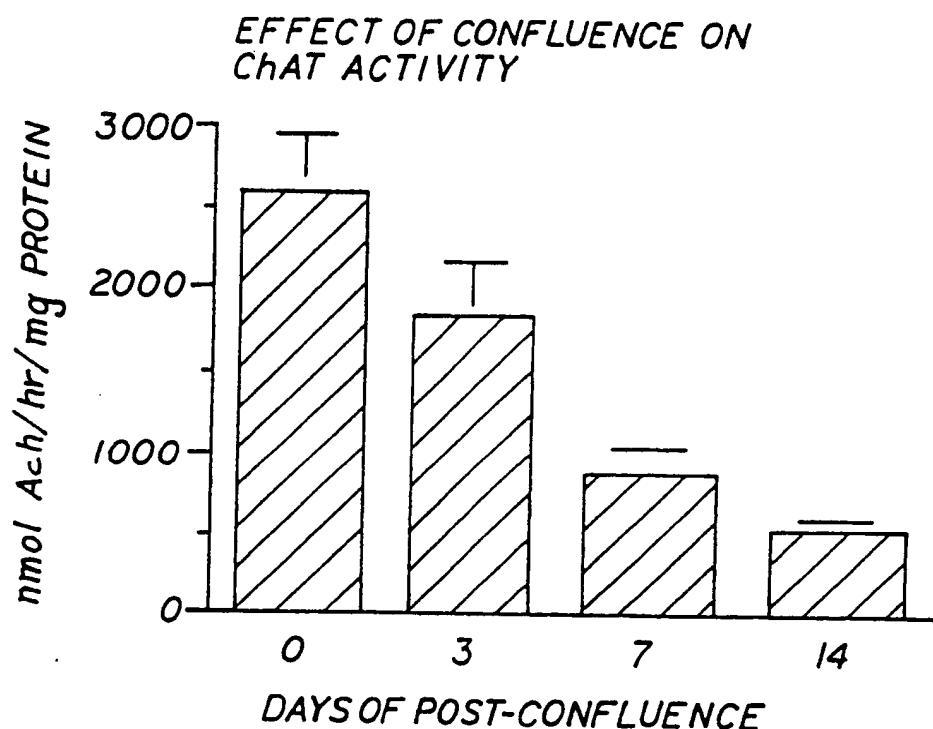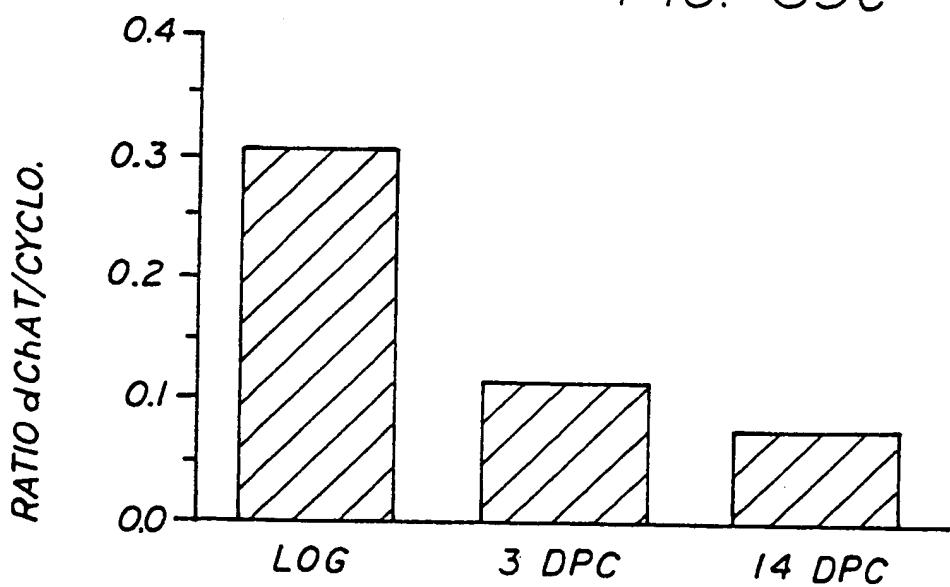
EFFECT OF CHOLINE ON QUIESCENT FIBROBLASTS

☒ INTRACELLULAR
☐ SECRETED
☒ TOTAL

% OF CONTROLS

7DPC   7DPC+CHOLINE

FIG. 41

pSV232ACAT
(5.34kb)

EcoRI(I)
PstI(206)
ApaI(476)
An
ScaI(1725)
BalI(1868)
CAT
EcoRI(2138)
HindIII(2383)
An An SV40
BamHI(2583)
(Bam/Bcl)(2820)
(Bam/Bcl)(3057)
Amp
Bglt(4460)
PstI(4585)
ScaI(4822)

FIG. 42

FIG. 43

FIG. 44

FIG. 45a

FIG. 45b

FIG. 46

SUBSTITUTE SHEET
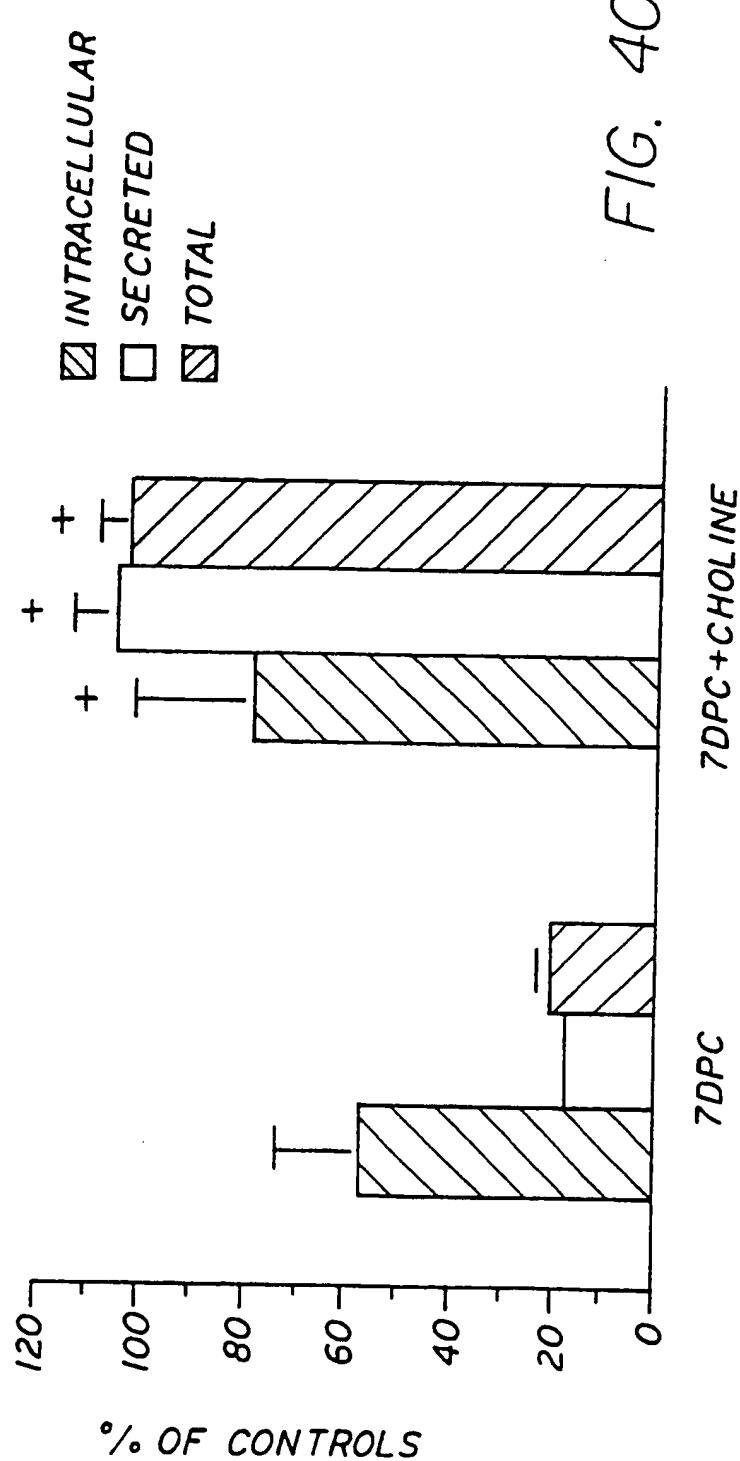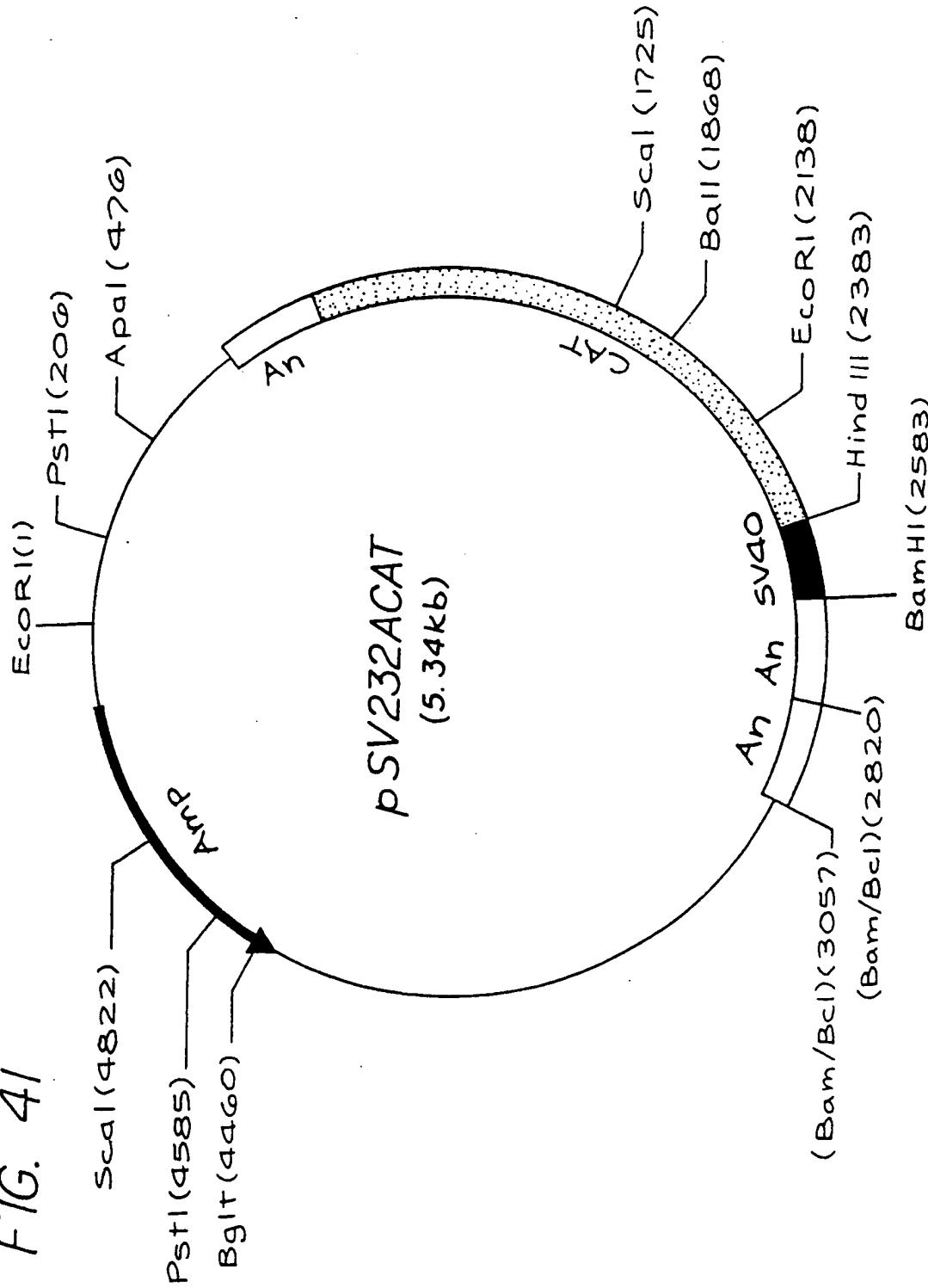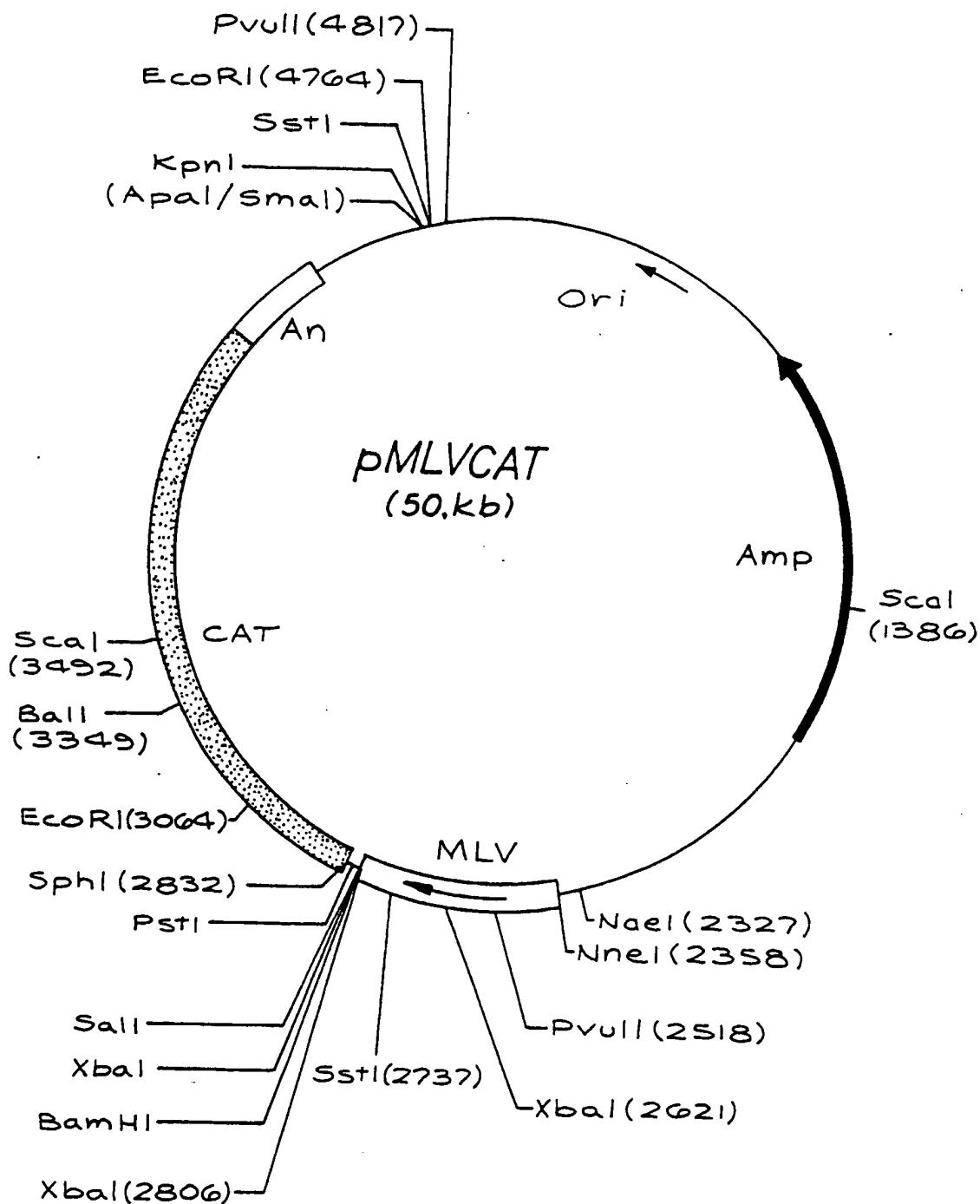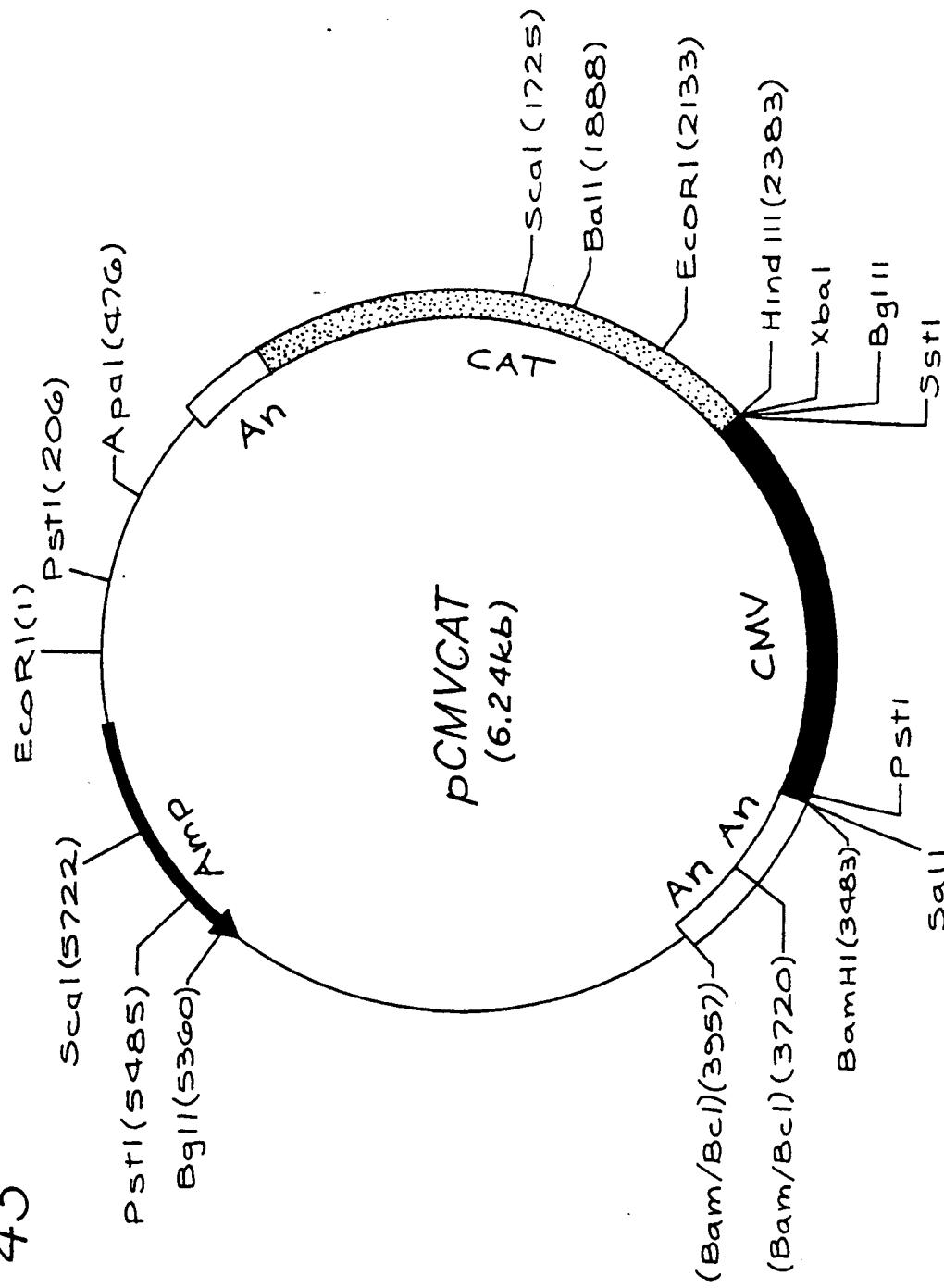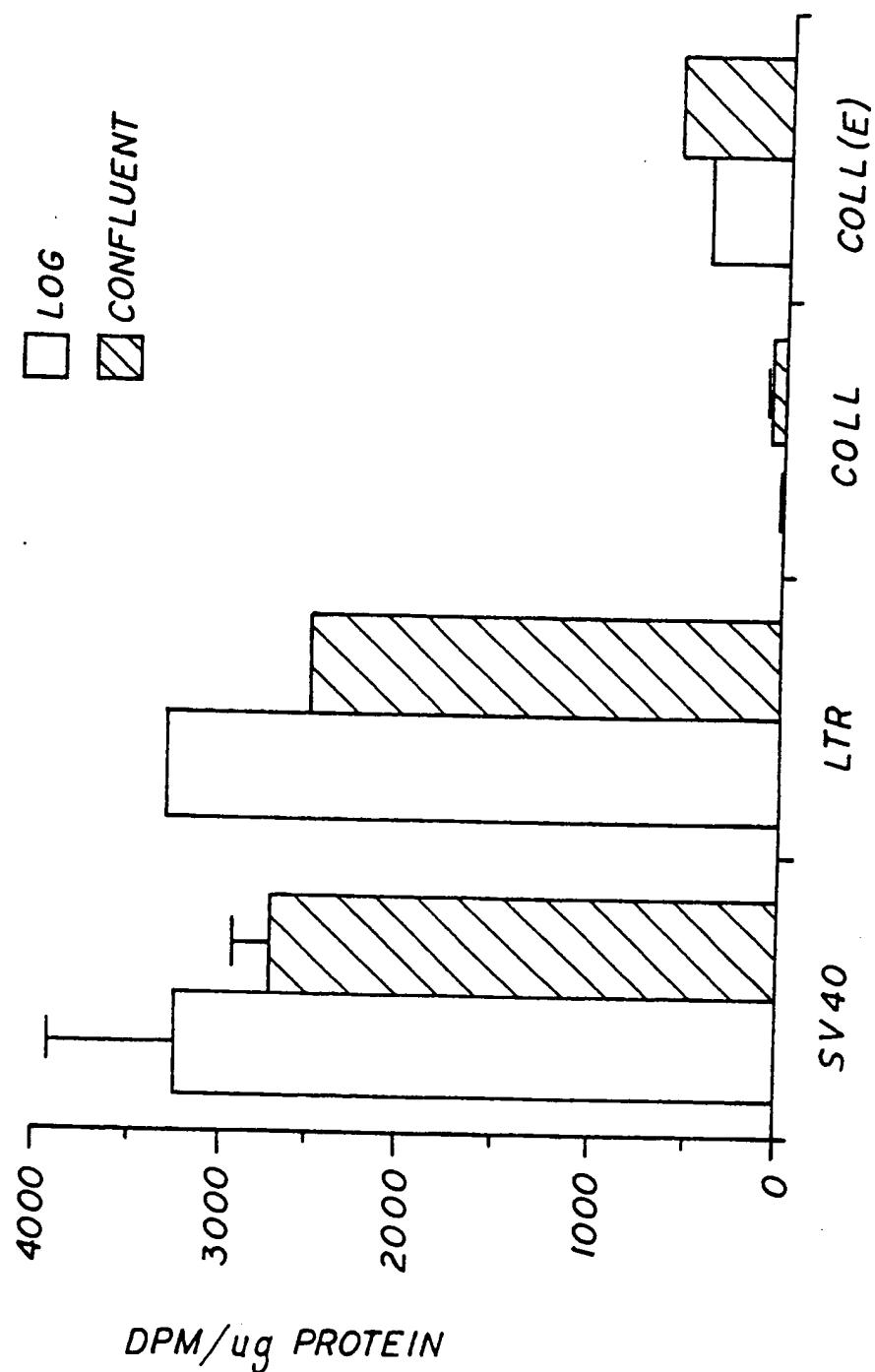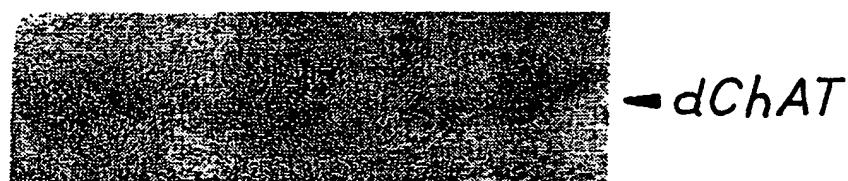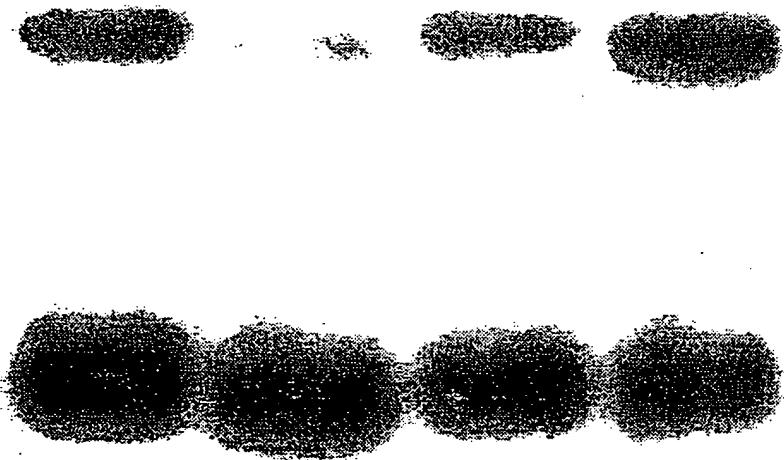
FIG. 47a

EFFECT OF SERUM ON COLLAGEN PROMOTER
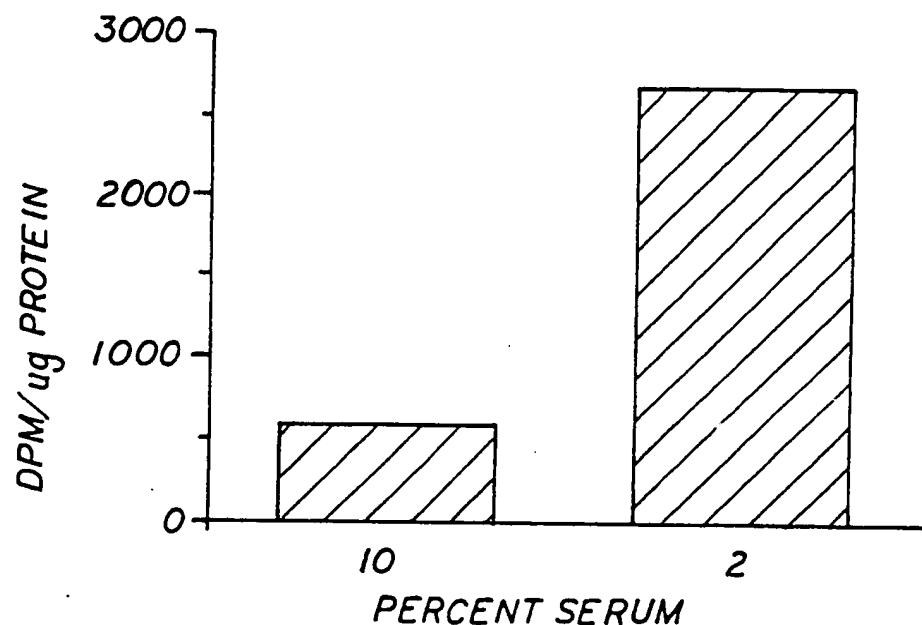
FIG. 47b

EFFECT OF CYTOKINES ON COLLAGEN PROMOTER
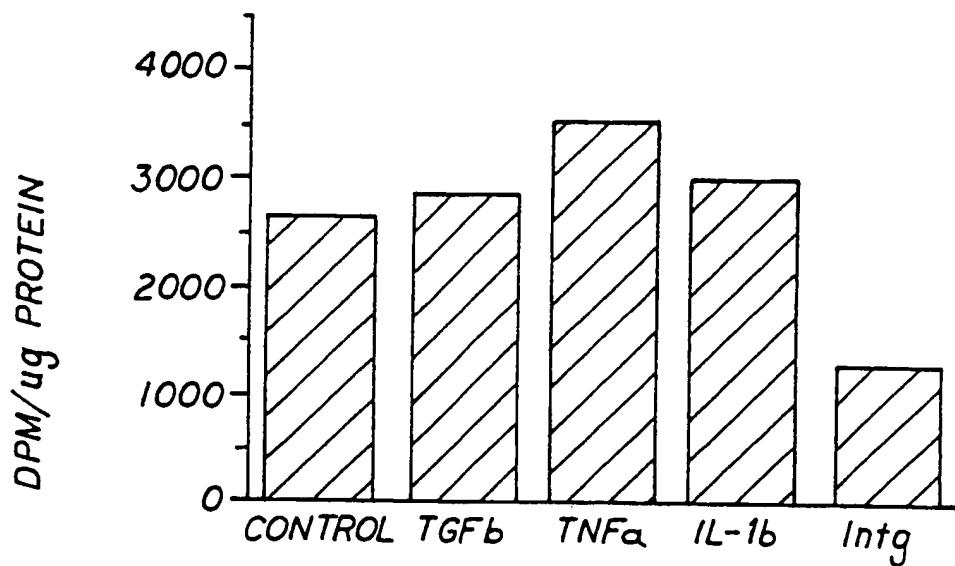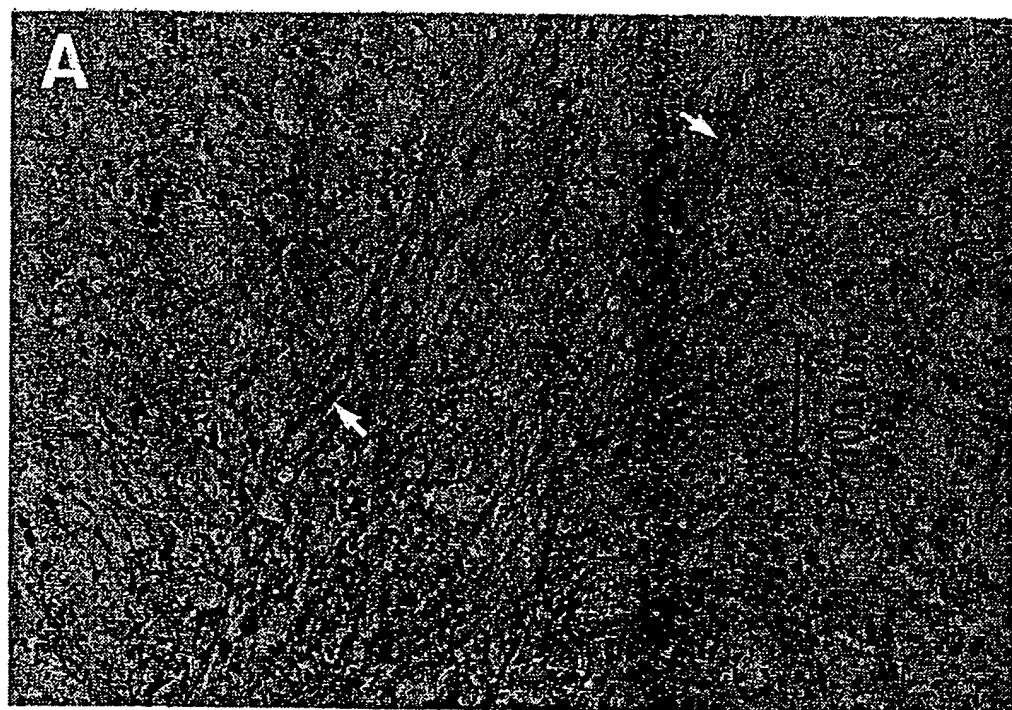
FIG. 48a
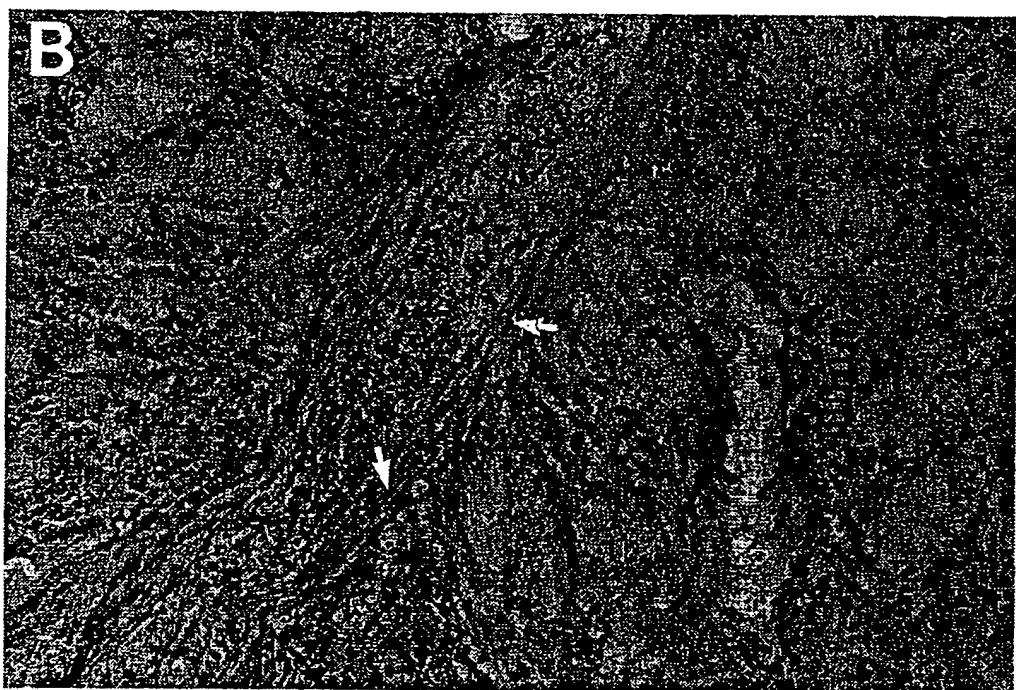
FIG. 48b
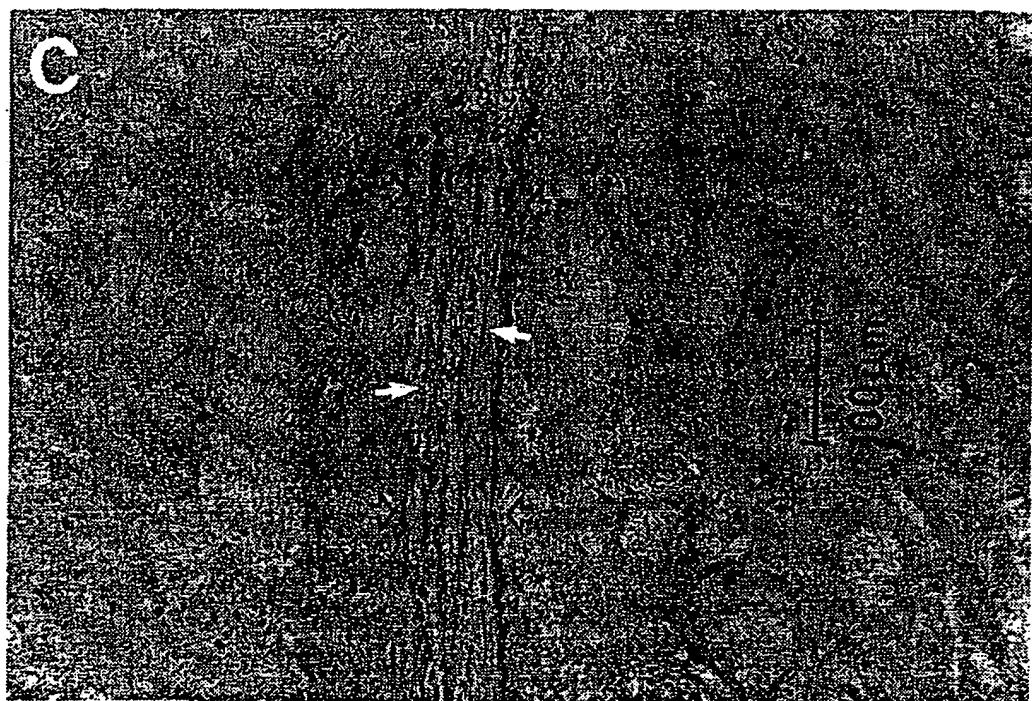
FIG. 48c
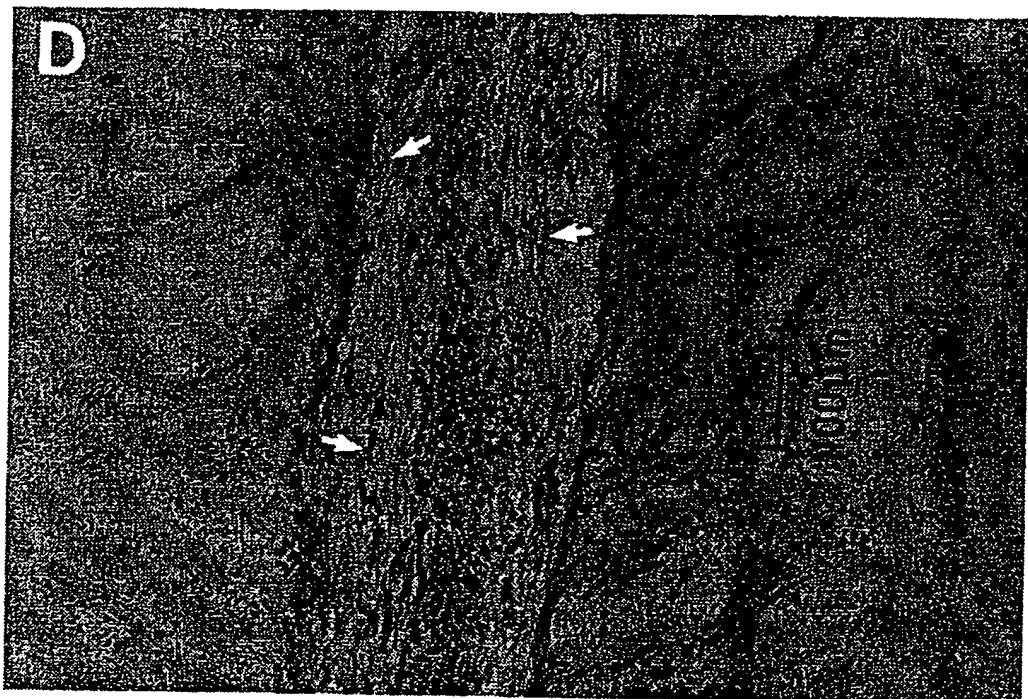
FIG. 48d

SUBSTITUTE SHEET
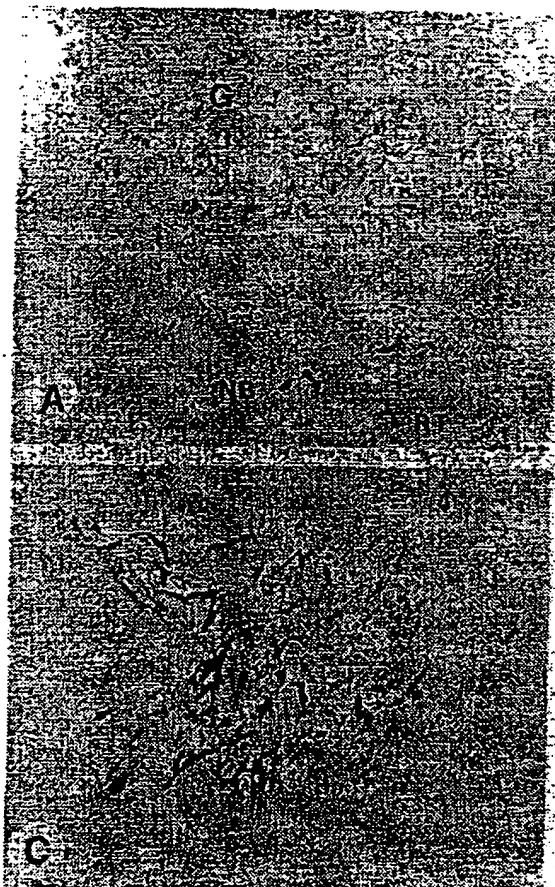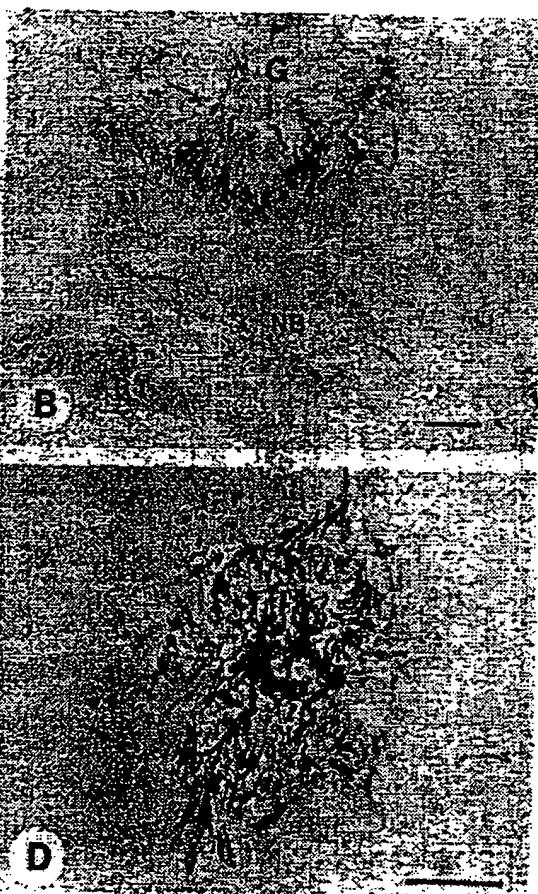
FIG. 49a

FIG. 49b

FIG. 49c

FIG. 49d

FIG. 50

FIG. 51
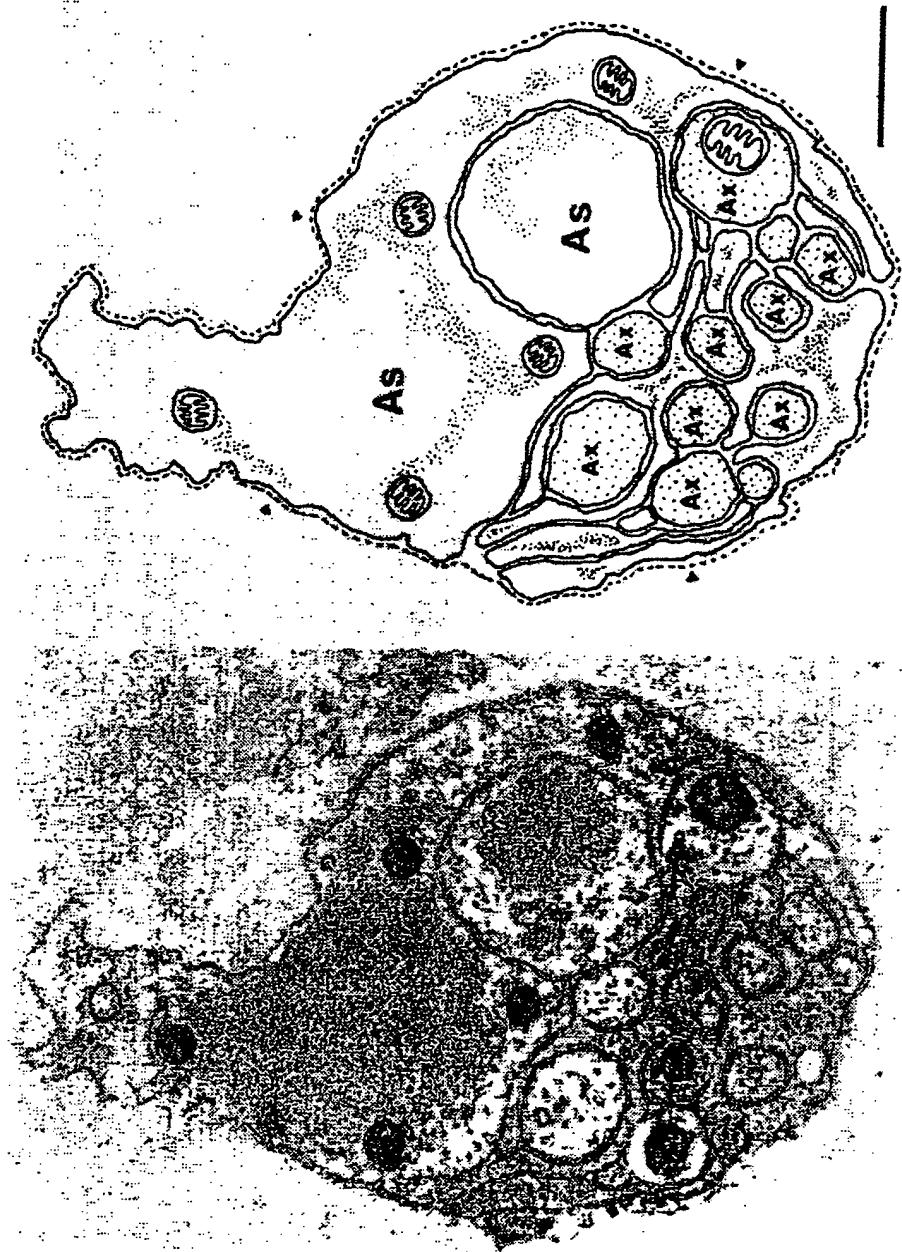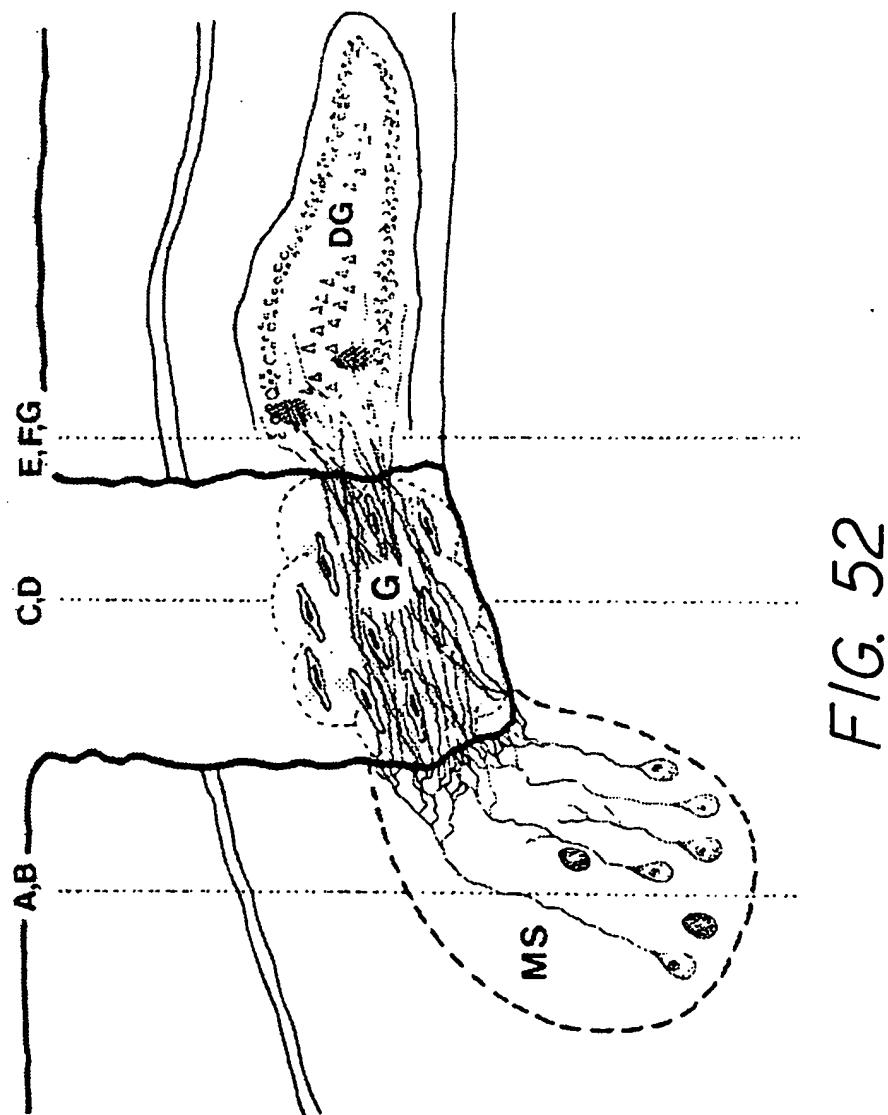
FIG. 52

FIG. 52a

FIG. 52c

FIG. 52b
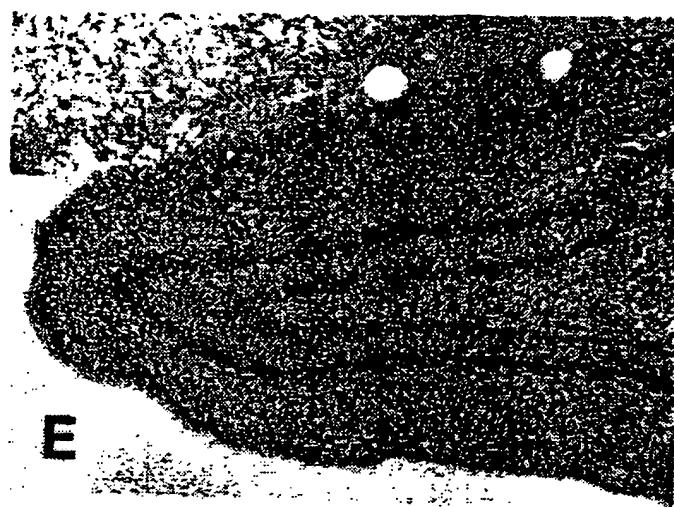
FIG. 52d

FIG. 52e

FIG. 52f

FIG. 52g

FIG. 53a   FIG. 53b
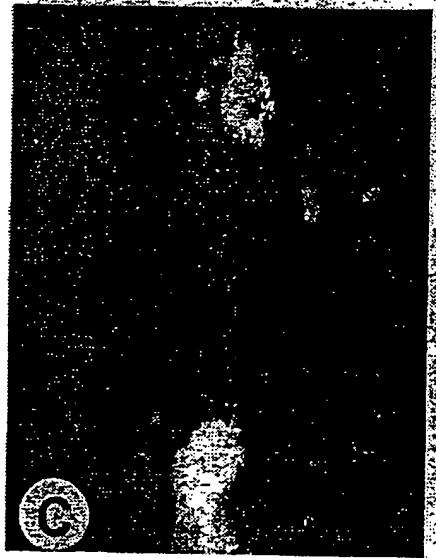
FIG. 53c   FIG. 53d
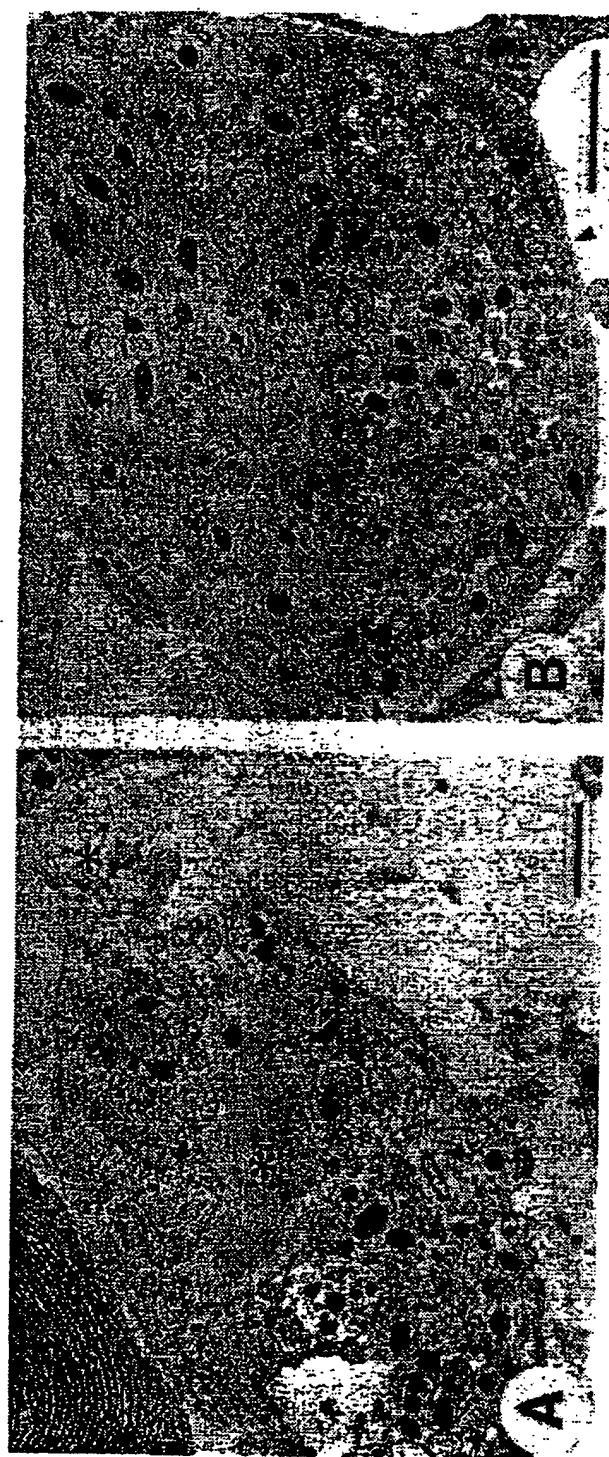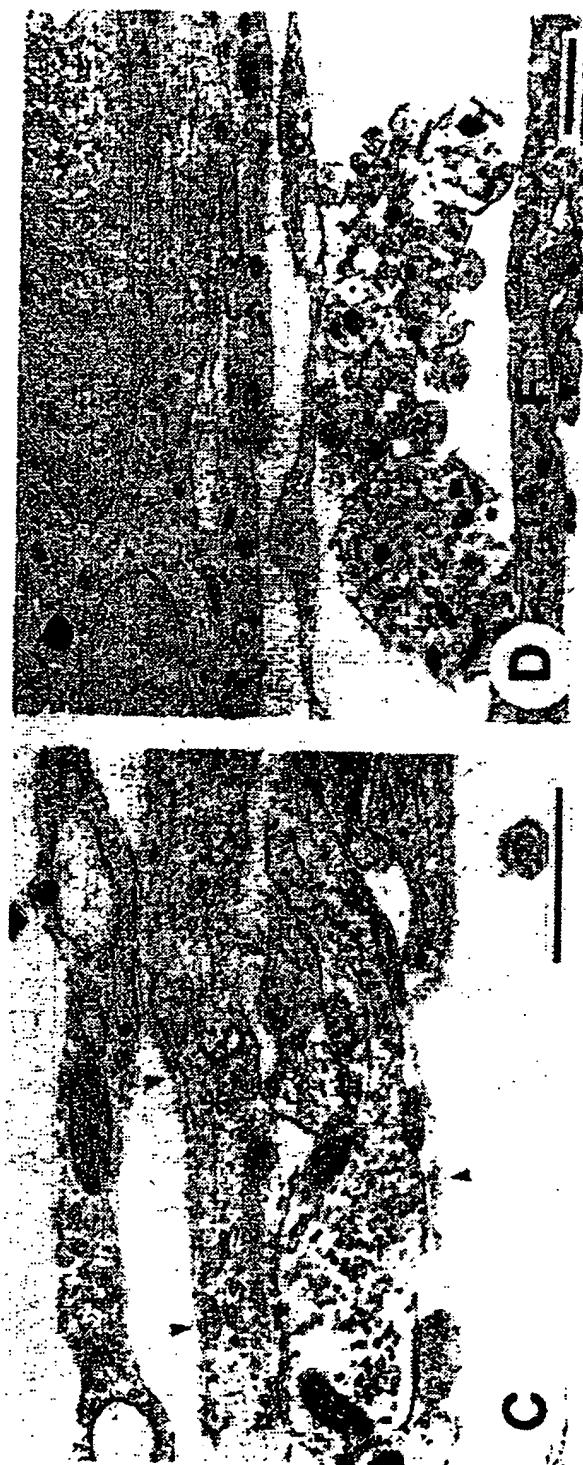
FIG. 54b

FIG. 54a

FIG. 54d

FIG. 54c

FIG. 54f
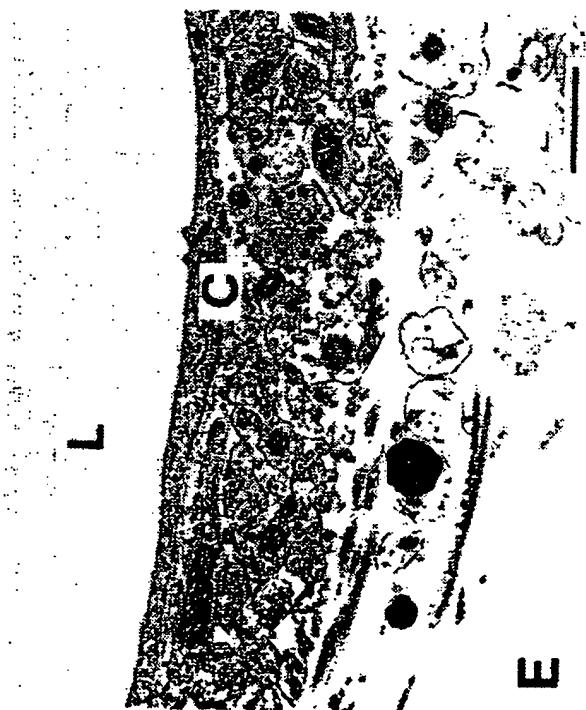
FIG. 54e

FIG. 55c
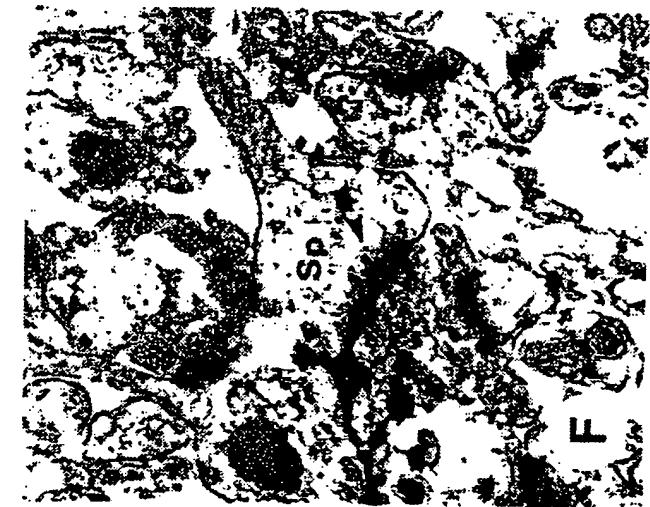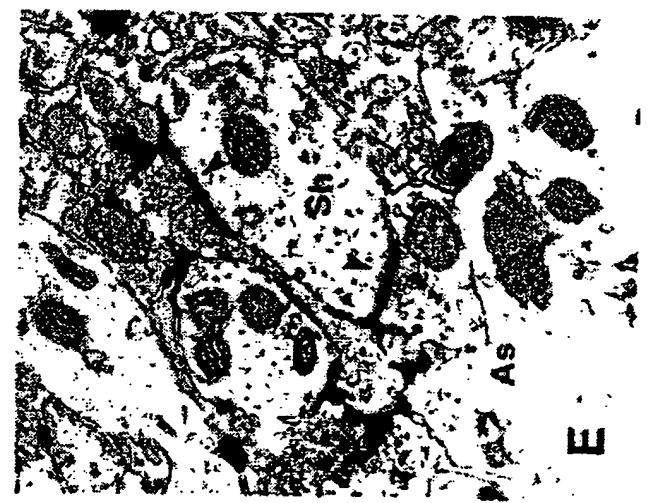
FIG. 55b

FIG. 55a

FIG. 55f

FIG. 55e

FIG. 55d

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/12;   A61K48/00;   C12N15/86;   C12N15/52

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K ; C07K ; C12N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[0]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO,A,9 006 757 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 28 June 1990 see the whole document	1-32,42, 43,48, 50,52, 67-71
Y	see the whole document	39-41, 44,45, 57-60
X	WO,A,8 902 468 (WHITEHEAD INSTITUTE) 23 March 1989 see page 4, line 16 - page 6, line 29	1
X,P	EP,A,0 474 979 (AMERICAN CYANAMID COMPANY) 18 March 1992 see page 5, line 20 - line 38; claim 11	1

−/−−

[0] Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
02 MARCH 1993	18. 03. 93

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	CHAMBONNET F.J.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X,P	WO,A,9 209 688 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 11 June 1992 see claims	1,73
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 83, February 1986, WASHINGTON US pages 725 - 729 KHILLAN,J.S. ET AL. 'Developmental and tissue-specific expression directed by the alpha 2 type I collagen promoter in transgenic mice' see the whole document	39-41, 44,45, 57-60

Box I	Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: **34-38,55,56,74 (see**
because they relate to subject matter not required to be searched by this Authority, namely:

, PCT-Rule 69.1(i)) . Claims 61-66 as far as they concerns a method of treatment sensu-stricto.
Remark: Although claims 1-33,39-54,57-60,67-73 and partially 61-66 are directed to a method of treatment of the human/animal body the search has

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

been carried out and based on the alleged effects of the compound/composition.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

US   9209896
SA   67288

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.   02/03/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9006757	28-06-90	US-A-	5082670	21-01-92
		CA-A-	2005567	15-06-90
		EP-A-	0449948	09-10-91
		JP-T-	4503351	18-06-92
WO-A-8902468	23-03-89	EP-A-	0378576	25-07-90
		JP-T-	3500124	17-01-91
EP-A-0474979	18-03-92	AU-A-	8258491	27-02-92
WO-A-9209688	11-06-92	AU-A-	9076191	25-06-92